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For consideration as a Brief Communication

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**Acidotolerant soil nitrite oxidiser ‘*Candidatus Nitrobacter laanbroekii*’ NHB1
4 alleviates constraints on growth of acidophilic soil ammonia oxidisers**

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Abstract

28 *Nitrobacter* strain NHB1 is a nitrite-oxidising bacterium previously co-enriched with the
neutrophilic ammonia-oxidising bacterium *Nitrosospira* AHB1, a consortium that nitrifies
30 under acidic conditions. Here we characterise the growth of isolated *Nitrobacter* strain
NHB1 as a function of pH and nitrite (NO_2^-) concentration, and its influence on the activity
32 of acidophilic soil ammonia-oxidising archaea (AOA) in co-culture. NHB1 is acidotolerant
and grows optimally at pH 6.0 (range 5.0 – 7.5) at initial NO_2^- concentrations of 500 μM .
34 However, the optimum decreases to pH 5.0 at lower initial NO_2^- concentrations closer to
those found in soil, with detectable growth down to pH 3.5. NHB1 has a comparatively
36 high affinity for NO_2^- with an apparent-half-saturation constant (54 μM) one order of
magnitude lower than its closest relative, the neutrophilic strain *Nitrobacter hamburgensis*
38 X14. In co-culture, NHB1 enhances the growth of acidophilic AOA. Specifically,
Nitrosotalea devaniterrae Nd1 and *Nitrosotalea sinensis* Nd2 are sensitive to NO_2^- -
40 derived compounds and only oxidise ~200-300 μM ammonia (NH_3) in batch cultures.
However, in co-culture with NHB1, pH ranges were lowered by ~0.5 pH units and both
42 strains could oxidise up to 2.7-2.9 mM NH_3 , only limited by buffering capacity. NHB1
possesses a cyanase facilitating reciprocal cross-feeding via generating cyanate-derived
44 NH_3 and utilising AOA-derived NO_2^- . Removal of NO_2^- is likely crucial for nitrifier growth
in acidic soils and this study highlights the importance of considering substrate and
46 metabolic product concentrations when characterising physiology. Genome analysis
reveals that NHB1 is distinct from validated species and the name '*Nitrobacter*
48 *laanbroekii*' is proposed.

50 Keywords

Nitrobacter, nitrite-oxidising bacteria, ammonia-oxidising archaea, acidophilic, acidic
52 soil, ammonia oxidation, nitrite oxidation, nitrification, cyanase

Main text

54 Microbially-mediated nitrification in soil is typically dominated by
chemolithoautotrophs. Canonical ammonia-oxidising archaea (AOA) and bacteria
56 (AOB) can perform the first step whereby ammonia (NH_3) is oxidised to nitrite (NO_2^-)
and is coupled to the activity of canonical nitrite-oxidising bacteria (NOB) which
58 subsequently oxidise NO_2^- to nitrate (NO_3^-). In acidic soils, ammonia oxidation activity
was considered paradoxical due to the reduced availability of NH_3 (pK_a for $\text{NH}_3:\text{NH}_4^+$
60 = 9.25). In addition, ammonia oxidisers (AO) are sensitive to NO_2^- -derived nitrous acid
(HNO_2) and associated decomposition products formed at low pH [1], necessitating
62 the use of buffers or neutralising media to sustain growth. The subsequent discovery
and cultivation of acidophilic and acidotolerant AO explained this paradox to a large
64 extent [2–5], but mechanisms including ureolytic activity [6, 7] or growth within biofilms
[8] or aggregates [9] also facilitate growth of neutrophilic nitrifiers under acidic
66 conditions. As canonical AO are present in environments together with NOB, enabling
mutualistic interactions [9, 10], the cooperative activity of NOB in soil is likely crucial
68 for AO activity.

Nitrobacter populations are a major component of soil NOB communities. While
70 most isolates are grown at neutral pH [11], acidophilic/acidotolerant strains have been
reported [9, 12]. These include *Nitrobacter* NHB1 which was originally cultivated from
72 pH 3.8 fertilised heathland soil together with the AOB *Nitrosospira* AHB1 [13]. Although
neutrophilic, AHB1 was active down to pH 4 when grown with NHB1 [14] and
74 neutrophilic *Nitrosospira*-like bacteria enriched from acidic forest soil grew at pH 4
when surrounded by *Nitrobacter*-like bacteria in aggregates [9]. Acidotolerant NOB
76 may therefore have a role in protecting ammonia oxidisers in acidic soils by removing
 NO_2^- before abiotic conversion to toxic compounds.

78 In this study we investigated the cell structure, genome content and growth
characteristics of the isolate NHB1, including the effect of pH on its growth and a
80 potential influence of NO_2^- concentration in defining its pH range. As isolated
acidophilic AOA are particularly sensitive to NO_2^- -derived compounds in culture, we
82 tested the hypothesis that NHB1 will positively impact the growth characteristics of
Nitrosotalea strains in co-culture via continuous nitrite removal, as typically occurs in
84 soil.

86 *Isolation and characterisation of NHB1*

NHB1 was isolated from a cryopreserved co-culture of NHB1 and AHB1 by substituting
88 NH_4Cl in the growth medium with NaNO_2 (Supplementary Methods). NHB1 is rod-
shaped with typical stacked intracytoplasmic membranes (Fig. 1a) and possesses a
90 3.3 Mb chromosome and two plasmids (0.28 Mb and 0.21 Mb). The closest validated
relative is the neutrophile *Nitrobacter hamburgensis* X14 [15] (Fig. 1b), sharing 99, 97,
92 and 98% identity with 16S rRNA, nitrite oxidoreductase sub-unit alpha (NxrA) and beta
(NxrB) encoding genes, respectively. High percentage identity of marker genes
94 between *Nitrobacter* species is typically observed [16] and contrasts with NHB1 having
a 24% smaller genome than *N. hamburgensis* X14 and sharing an average nucleotide
96 identity of 92.4%. We therefore propose the following candidate species: *Nitrobacter*
laanbroekii sp. nov. (laan.broek'i.i. N.L. gen. n. *laanbroekii*), named in honour of the
98 Dutch microbiologist Hendrikus J. Laanbroek who was involved in its original
cultivation and has made valuable contributions to understanding the ecology of NOB.
100 In addition to expected core genes for carbon and energy metabolism, NHB1
possesses genes for dissimilatory sulphur oxidation, assimilatory nitrite reductase and
102 utilisation of carbon monoxide as found for other *Nitrobacter* species [16].

Growth occurred with initial NO_2^- concentrations up to >5 mM (inhibited at ~10
104 mM) and temperatures ranging 10-30°C, with a maximum specific growth rate (μ_{max})
of 1.17 d⁻¹ (s.e.=0.05) at 25°C and 0.5 mM (Fig. 1c and 1d). As indicated from its
106 previous enrichment, NHB1 is acidotolerant but μ_{max} at different pH varied with initial
 NO_2^- concentration. At 500 μM , growth occurred at pH 5.0-7.5 with optimal growth at
108 pH 6.0 (Fig. 1e), but reduction of initial NO_2^- concentration to 100 μM decreased
optimal growth pH to 5.5 (μ_{max} 0.93 d⁻¹ (s.e.=0.01)) with further reductions in pH growth
110 optimum to 5.0 at 50 and 20 μM (μ_{max} 0.77 d⁻¹ (s.e.=0.03) and 0.70 d⁻¹ (s.e.=0.01),
respectively). At the lowest NO_2^- concentration, the limit for growth was extended to
112 pH 3.5 (μ_{max} 0.26 d⁻¹ (s.e.=0.01)). Whole cell nitrite oxidation kinetics at 25°C and pH
6 were determined using substrate concentration-dependent oxygen
114 microrespirometry (Fig. S1). An apparent-half-saturation concentration ($K_{m(\text{app})}$) of 54
 μM NO_2^- (s.e.=7) was higher than several canonical nitrite-oxidizing *Nitrospira* but
116 within the lower range of those observed for other *Nitrobacter* strains, and one order
of magnitude lower than *N. hamburgensis* X14 (544 μM) [17] (Fig. 1f).

118

Influence of NHB1 on acidophilic AOA physiology

120 *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 are two AOA strains with
pH optima ~5.0 [2]. Although obligately acidophilic with adaptations to low pH, both
122 are sensitive to NO_2^- with concentrations as low as 10 μM reducing growth rates [2].
In standard batch cultures with 500 μM NH_4^+ and pH 5.2, yields of NO_2^- are typically
124 200-300 μM (Fig. 2a) and one to two orders of magnitude lower than neutrophilic soil
AOA [18, 19]. However, in contrast to growth in isolation, all 500 μM NH_4^+ was oxidised
126 when grown in co-culture with NHB1 (Fig. 2b). The amount of NH_4^+ oxidised could be
extended to 1.2 and 2.7-2.9 mM for both strains when 2-(N-morpholino)ethanesulfonic
128 acid (MES) buffer concentrations were increased to 20 and 50 mM, respectively (Fig.

2c, Fig. S2), with growth inhibited when pH eventually decreased to <3.8 (Fig. S2). In
130 the presence of NHB1, the pH range of both strains was extended down by ~0.5 pH
units and μ_{max} increasing significantly at pH ≤ 5.0 ($P < 0.05$) (Fig. 2d). NHB1 possesses
132 genes encoding enzymes that were previously demonstrated to facilitate mutualistic
interactions with AO, including cyanase which produces NH_4^+ from cyanate and
134 enables reciprocal cross-feeding [10]. While cyanate abiotically degraded rapidly to
 NH_4^+ in pH 5.2 medium (Fig. S3), NHB1 cyanase activity was the dominant
136 mechanism generating ammonium at pH 6.0 in cultures supplemented with 0.05 or
0.5 mM cyanate (Fig. 2e). Although ≥ 0.5 mM cyanate can support other AOA or
138 nitrifying co-cultures [10], this concentration inhibited both *Nitrosotalea* strains, with
less than 5% of NOB-generated ammonium being oxidised through to NO_3^- and with
140 no accumulation of NO_2^- (Fig. S4). However, Nd1 and Nd2 used the majority or all
cyanate-derived NH_4^+ at 0.05 mM, respectively, a concentration higher than that
142 typically found in soil where cyanase is continuously turned over [20].

144 *Summary*

'*Ca. Nitrobacter laanbroekii*' NHB1 is an acidotolerant NOB isolated from acidic soil
146 with a relatively high affinity for NO_2^- . Growth in acidophilic consortia demonstrates
that continued removal of NO_2^- in acidic soil is likely crucial for sustained growth of AO
148 and that consideration of substrate and metabolic product concentrations is essential
when characterising physiology.

150

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160

Conflict of interest statement

162 The authors declare no conflicts of interest.

Data availability

The genome sequence of '*Ca. Nitrobacter laanbroekii*' NHB1 is available under NCBI
166 BioProject accession number PRJNA1074292.

168

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228 **Figure legends**

Figure 1. Characterisation of the isolated strain 'Ca. Nitrobacter laanbroekii' NHB1.

230 (A) Transmission electron micrograph of cells in lateral and longitudinal orientation.
Scale bars represent 0.2 μm . (B) Maximum likelihood phylogenomic tree of NHB1,
232 selected *Nitrobacter* isolates and outgroup reference *Rhodopseudomonas palustris*
(also of the *Nitrobacteraceae*) using 11,751 unambiguously aligned amino acid
234 positions inferred from 63 single copy genes. The scale bar represents an estimated
0.01 changes per position and values at nodes describe percentage bootstrap support
236 (100 replicates). The influence of (C) temperature, (D) initial nitrite concentration, and
(E) pH on maximum specific growth rates (μ_{max}) were determined with mean values
238 and standard errors (mostly smaller than symbol size) from triplicate cultures plotted.
(F) Apparent half-saturation constant ($K_{m(app)}$) for NO_2^- of 'Ca. Nitrobacter laanbroekii'
240 NHB1 (mean value of four replicates) and various *Nitrobacter*, *Nitrotoga* and *Nitrospira*
strains (Supplementary Methods).

242

Figure 2. Influence of 'Ca. Nitrobacter laanbroekii' NHB1 on growth characteristics of

244 acidophilic AOA *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 in batch
culture. Mean values and standard errors (mostly smaller than symbol size) from
246 triplicate cultures are plotted in each panel. (A) Growth of Nd1 and Nd2 grown in
isolation and supplied with 0.5 mM NH_4^+ . (B) Growth of Nd1 and Nd2 in co-culture with
248 NHB1 supplied with 0.5 mM NH_4^+ . (C) NO_3^- production by co-cultures of Nd1 or Nd2
with NHB1 in medium with MES buffer concentration increased from 10 mM to 50 mM
250 and 0.5, 1, 2, 3, 4, 5 or 10 mM NH_4^+ . (D) The influence of NHB1 on the maximum
specific growth rates (μ_{max}) of Nd1 and Nd2 compared to growth in isolation at different
252 pH. An asterisk highlights a significant difference in μ_{max} ($p = <0.05$) for an individual
pH (calculated using Student's t-test). (E) Reciprocal cross-feeding at pH 6.0 between

254 NHB1 and Nd1 or Nd2 with 50 μM cyanate supplied as an NH_4^+ source. NH_4^+ , NO_2^-
and NO_3^- concentrations were determined for all cultures. Decomposition of cyanate
256 in the abiotic control produced $<3.8 \mu\text{M}$ NH_4^+ during the period of incubation.

258

Figure 1

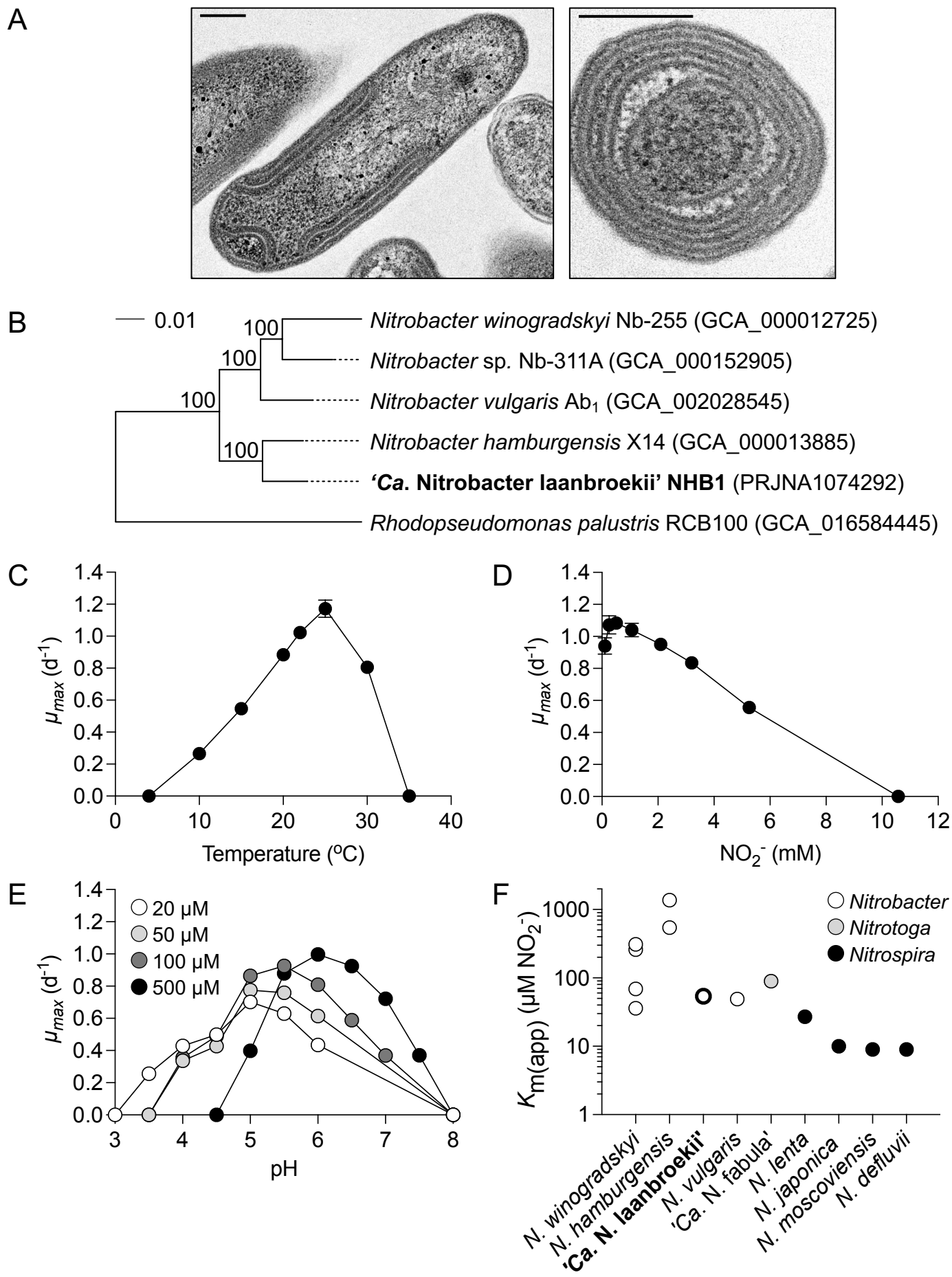
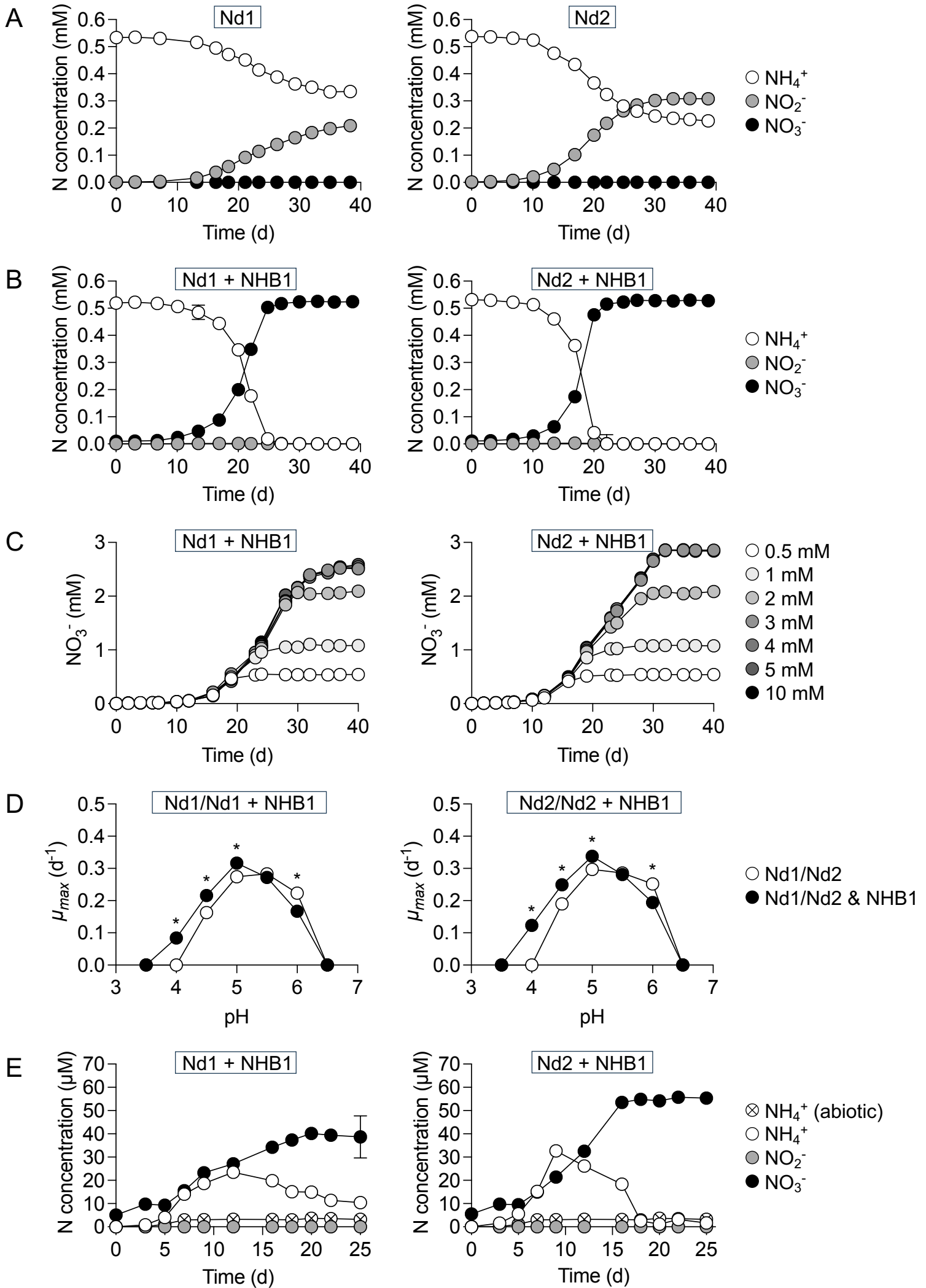


Figure 2



Supplemental Material

2

Materials and methods

4 *Isolation and maintenance of 'Ca. Nitrobacter laanbroekii' NHB1 in culture*

The co-culture of *Nitrosospira* sp. AHB1 and 'Ca. N. laanbroekii' NHB1 was obtained from cultures established in 1988 using pH 3.8 heathland soil (Hoorneboeg; 52°15'N, 5°10'E) under *Calluna vulgaris* (Heather) and *Deschampsia flexuosa* (Wavy hair-grass) vegetation [1, 2]. Cells from a cryostock were resuscitated by growing in 50 ml unbuffered medium with 2.5 mM NH₄⁺ adjusted to pH 7. Specifically, the medium contained (L⁻¹): KH₂PO₄ (0.1 g); NaCl (0.5 g); MgSO₄·7H₂O (0.04 g); CaCl₂·2H₂O (0.02 g); (NH₄)₂SO₄ (0.33 g); FeSO₄·7H₂O (2.46 mg); NaMoO₄·2H₂O (0.1 mg); MnCl₂ (0.2 mg); Na₂EDTA (3.31 mg); ZnSO₄·7H₂O (0.1 mg), CuSO₄·5H₂O (20 µg), and CoCl₂ (2 µg) [1]. Cultures were incubated in the dark at 25°C without shaking. The same medium was used to purify 'Ca. N. laanbroekii' NHB1, except the pH was reduced to 5.5 and (NH₄)₂SO₄ replaced with 500 µM NaNO₂ (0.035 g L⁻¹). Isolation was achieved by routine transfer (approximately weekly) of 1 ml exponentially growing culture into 50 ml fresh medium. The purity of 'Ca. N. laanbroekii' NHB1 was initially confirmed by the absence of ammonia oxidation activity when inoculated into fresh (NH₄)₂SO₄-containing medium, loss of the band corresponding to the 16S rRNA gene amplicon of *Nitrosospira* sp. AHB1 in denaturing gradient gel electrophoresis (DGGE) analysis, and phase contrast microscopy (data not shown). Purity was confirmed by the absence of any contaminating DNA during genome sequencing and assembly.

The suitability of an acidophilic ammonia-oxidising archaea (AOA) medium for growing 'Ca. N. laanbroekii' NHB1 in potential co-culture experiments was assessed using NO₂⁻ instead of NH₄⁺ as an energy source. Specifically, acidic 'freshwater medium' (FWM) [3] consisted of NaCl (1 g L⁻¹), MgCl₂ (0.4 g L⁻¹), CaCl₂ (0.1 g L⁻¹),

KH₂PO₄ (0.2 g L⁻¹), KCl (0.5 g L⁻¹), 1 ml modified non-chelated trace element solution
28 [4], 1 ml 7.5 mM NaFeEDTA, 2 mM NaHCO₃, and 500 μM NaNO₂ (replacing 500 μM
NH₄Cl), was buffered with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and
30 adjusted to pH 5.5 before sterilisation by filtration using a GL45 bottle-top 0.2 μm filter
unit (Nalgene, Rochester, USA). Successful growth of '*Ca. N. laanbroekii*' NHB1 in
32 this medium resulted in its subsequent use for routine growth and maintenance.

34 *Ammonia, nitrite and nitrate concentration measurements*

All inorganic N concentrations were measured colorimetrically in 96-well plates using
36 50 μl of growth medium or adequate dilutions as described previously [5]. Briefly, NH₄⁺
concentration was estimated using the indophenol method [6]. NO₂⁻ and NO₃⁻
38 concentrations were estimated using a method modified from Shinn [7] and Doane
and Horwath [8]. NO₂⁻ was detected by adding 60 μl of diazotising reagent (2.2 mM
40 sulphanilamide in 3.3 M HCl) followed by 20 μl coupling reagent (0.12 mM N-(1-
naphthyl)-ethylenediamine in 0.12 M HCl). NO₂⁻ concentration was estimated
42 immediately before reducing NO₃⁻ to NO₂⁻ by adding 20 μl vanadium chloride solution
(4.5 mM vanadium(III) chloride in 1 M HCl), incubating for 90 min at 35°C in the dark
44 and measuring NO₂⁻ + NO₃⁻ concentration.

Characterisation of Ca. Nitrobacter laanbroekii NHB1

46 To determine temperature, pH and NO₂⁻ concentration range, triplicate 50 ml cultures
were statically incubated in sterile 100 ml culture bottles in the dark between 4 and
48 35°C, in medium adjusted to pH 3.0 to 8.0, and containing initial NO₂⁻ concentrations
between 20 μM and 10 mM, respectively. As NO₂⁻ is known to degrade abiotically,
50 particularly at pH lower than 5.5, triplicate sterile controls were also incubated with
each treatment. All cultures were inoculated with 1% (vol/vol) early stationary phase

52 culture that had consumed 500 μM NO_2^- . The growth of cultures was monitored via
regular assessment of NO_2^- concentrations. As NO_2^- was stoichiometrically converted
54 to NO_3^- by 'Ca. N. laanbroekii' NHB1 (Fig. S5), measurement of NO_3^- concentrations
was not performed on a regular basis, but NO_3^- production was calculated via NO_2^-
56 consumption after calculating the predicted abiotic degradation rate of NO_2^- ,
particularly under acidic pH (Fig. S6), enabling calculation of μ_{max} during the
58 exponential growth phase of the cultures (Fig. S7).

60 *Growth of Nitrosotalea strains in isolation or co-culture with 'Ca. Nitrobacter laanbroekii' NHB1*

62 Cultures of isolated *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 or
stable co-cultures of *N. devaniterrae* Nd1 or *N. sinensis* Nd2 with 'Ca. N. laanbroekii'
64 NHB1 were established by inoculating 1 ml of early stationary phase cultures into 50
ml standard FWM medium for acidophilic AOA (pH 5.2) containing 500 μM NH_4Cl in
66 sterile 100 ml culture bottles. While *N. devaniterrae* Nd1 and 'Ca. N. laanbroekii' NHB1
have similar temperature ranges and growth optima, *N. sinensis* Nd2 grows optimally
68 $\sim 35^\circ\text{C}$ [9] and above the temperature range of 'Ca. N. laanbroekii' NHB1. A
temperature of 25°C was therefore used for isolation vs co-culture experiments.
70 Growth of (co-)cultures was monitored via measurement of NH_4^+ , NO_2^- and NO_3^-
concentrations until stationary phase. The effect of pH and substrate concentration
72 was determined in 30 ml sterile plastic Universal bottles (Greiner Bio-One, Les Ulis,
France) containing 20 ml of medium. Co-cultures can be maintained indefinitely by
74 transfer (2% vol/vol) every 2-3 weeks into sterile medium.

In co-culture experiments to examine the potential cyanase activity of NHB1,
76 ammonium was substituted with 50 or 500 μM cyanate. In experiments examining
yields of NO_3^- with higher NH_4^+ concentrations, despite the absence of NO_2^- -derived

78 acids in co-culture, pH decreased with growth and higher concentrations of MES (both
20mM and 50 mM) were required to sustain growth after consumption of ~800 μM
80 NH_4^+ .

82 *Determination of substrate kinetics*

Cellular nitrite oxidation kinetics were determined from instantaneous substrate-
84 dependent oxygen uptake measurements as previously described using a multiple
injection method [10, 11]. Briefly, measurements were performed in a water bath at
86 25°C with a microrespirometry (MR) system, equipped with a PA2000 picoammeter
and a 500 μm tip diameter OX-MR oxygen microsensor (Unisense, Aarhus, Denmark),
88 polarized continuously for at least 24 h before use. Active NHB1 cells were taken from
early stationary phase cultures soon after substrate depletion or harvested and
90 concentrated (6000 x g, 10 min, 20 °C) from NO_2^- replete active cultures using Amicon
Ultra-15 10 kDa cut-off centrifugal filter units (Merck Millipore, Darmstadt, Germany).
92 Concentrated cells were washed with and resuspended in substrate-free medium (pH
6) prior to MR measurements. Whole cell activity rates of NHB1 were fit with the
94 Michaelis-Menten model to derive the apparent whole cell reaction half saturation
concentration ($K_{m(\text{app})}$; $\mu\text{M NO}_2^-$). The $K_{m(\text{app})}$ of 'Ca. N. laanbroekii' NHB1 was
96 compared with other nitrite oxidiser strains using previously published values [12–15]

98 *Transmission electron microscopy*

NHB1 cells were recovered from 1 L of late exponential culture by filtering onto a 0.2
100 μm mixed cellulose ester filter before resuspending cells in 2 ml fresh medium and
pelleting by centrifugation at 14,000 x g for 40 min. One volume (500 μl) 4%
102 glutaraldehyde (v/v) was added to ~500 μl medium overlying the pellet and stored at
4°C overnight. Cells were then post-fixed with 1% OsO_4 in 0.3 M cacodylate buffer (pH

104 7.4) for 1 h at 4°C before ethanol dehydration and transfer to propylene oxide. Cells
were embedded in Epon epoxy resin and inclusion obtained by polymerisation at 60°C
106 for 72 h. Ultra-thin sections (100 nm) were cut using a UC7 ultramicrotome (Leica,
Nanterre, France), mounted on 200 mesh copper grids (EMS, Hatfield, USA) and
108 contrasted with uranyl acetate and lead citrate. Sections were examined with a JEM-
1400 120 kV transmission electron microscope (Jeol, Tokyo, Japan) equipped with a
110 Orius 1000 camera (Gatan, Pleasanton, USA) in the wide-field position and Digital
Micrograph software (Gatan) at the Centre d'Imagerie Quantitative Lyon-Est,
112 Université Claude Bernard Lyon 1.

114 *Genome sequencing, assembly and annotation*

DNA was extracted from pelleted cells using a standard SDS buffer and phenol:
116 chloroform:isoamyl alcohol chemical lysis method [16] and sequenced using in-house
MiSeq (Illumina, San Diego, USA) and MinION (Oxford Nanopore, Oxford, UK)
118 platforms. A library for MiSeq paired-end sequencing was prepared using a Nextera
XT kit, according to the manufacturer's instructions (Illumina) and sequencing
120 performed with a V2 kit, producing 20.1 million reads with an average length of 243.4
bp after quality trimming performed using TrimGalore
122 (<https://github.com/FelixKrueger/TrimGalore>). For MinION sequencing, DNA was
sheared to approximately 8 kb using a g-TUBE (Covaris, Brighton, UK) and a library
124 prepared with the SQK-MAP006 kit, according to the manufacturer's instructions
(Oxford Nanopore). Sequencing on a MinION flow cell produced 39,064 reads with an
126 average length of 6,739 bp. The genome was assembled using Unicycler [17] with
default settings for both MinION and Illumina data. Gene prediction was performed
128 using Prodigal version 2.6.3 [18] and annotation performed using Diamond BLASTp
v0.8.36 (e-value 10^{-5}) [19] with the NCBI nr database release 244 [20]. Genome

130 quality was assessed using CheckM [21], estimating 99.1% completeness and 0.34%
contamination, and GToTree [22], detecting 100% of expected single copy genes with
132 0% redundancy. Average nucleotide identity (ANI) between different *Nitrobacter*
strains was assessed using reciprocal best hits (two-way ANI) ([http://enve-](http://enve-omics.ce.gatech.edu/ani/)
134 [omics.ce.gatech.edu/ani/](http://enve-omics.ce.gatech.edu/ani/)) [23].

136 *Phylogenomic analysis*

Single copy genes present in all compared genome sequences were identified and
138 aligned using GToTree [22] before manual refinement. Maximum likelihood analysis
was performed on unambiguously aligned concatenated protein sequences (11,751
140 amino acid positions inferred from 63 single copy genes) using PhyML [24] with
automatic model selection (Q.plant with FreeRate variation (four rates) across sites)
142 and bootstrap support (100 replicates).

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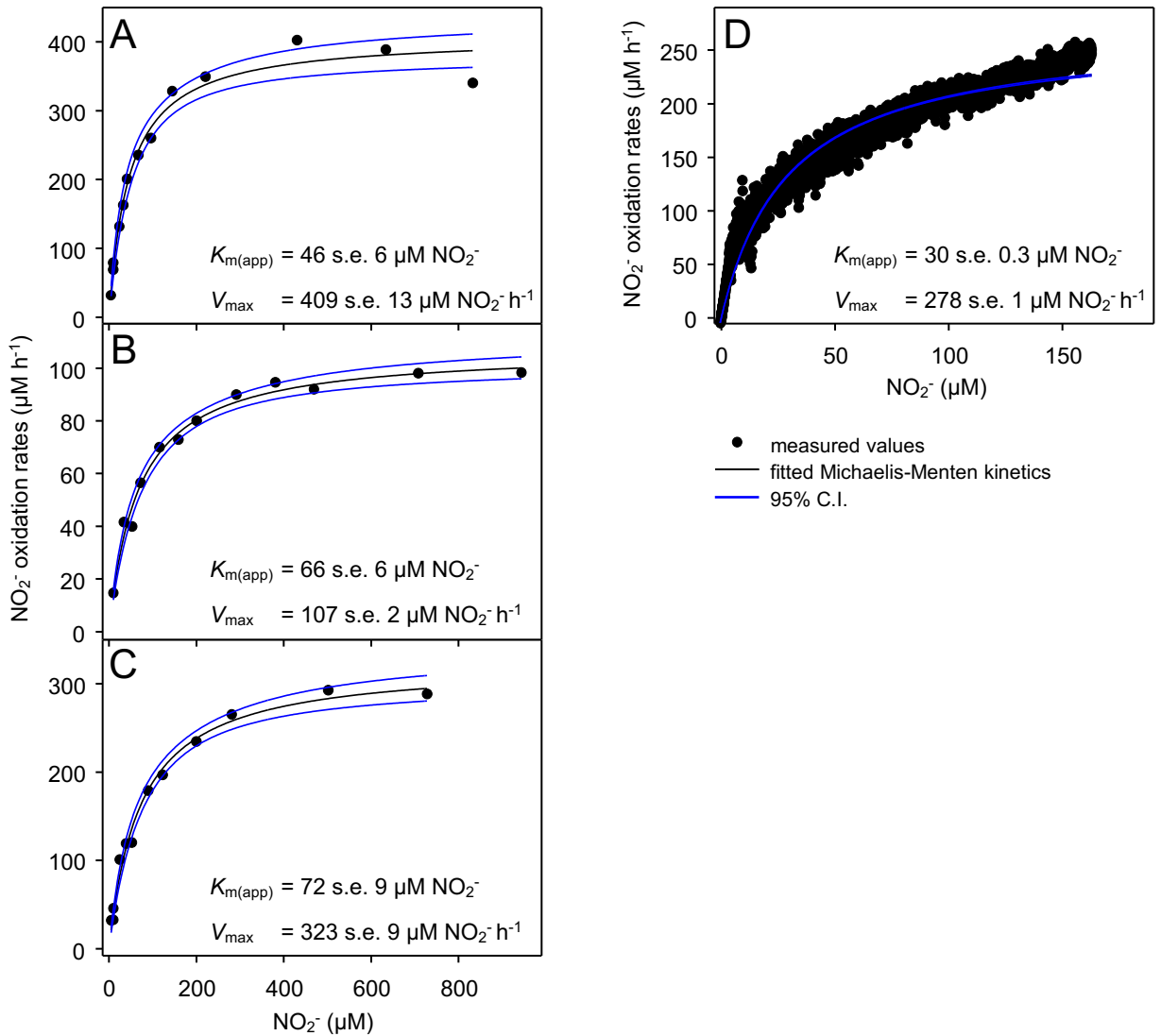


Figure S1. Apparent half-saturation ($K_{m(\text{app})}$) and maximum oxidation rates (V_{max}) for NO_2^- calculated using oxygen microrespirometry (MR) after fitting data of oxidation rate vs NO_2^- concentration to the Michaelis–Menten model with 95% confidence intervals (C.I.) plotted. Three experimental replicates measured oxidation rates after injection varying concentrations of NO_2^- (A,B,C) into the MR chamber and one replicate (D) used a single injection of 250 μM . Biomass was not measured and V_{max} was therefore not normalised to cell numbers or protein content and varies substantially amongst replicates.

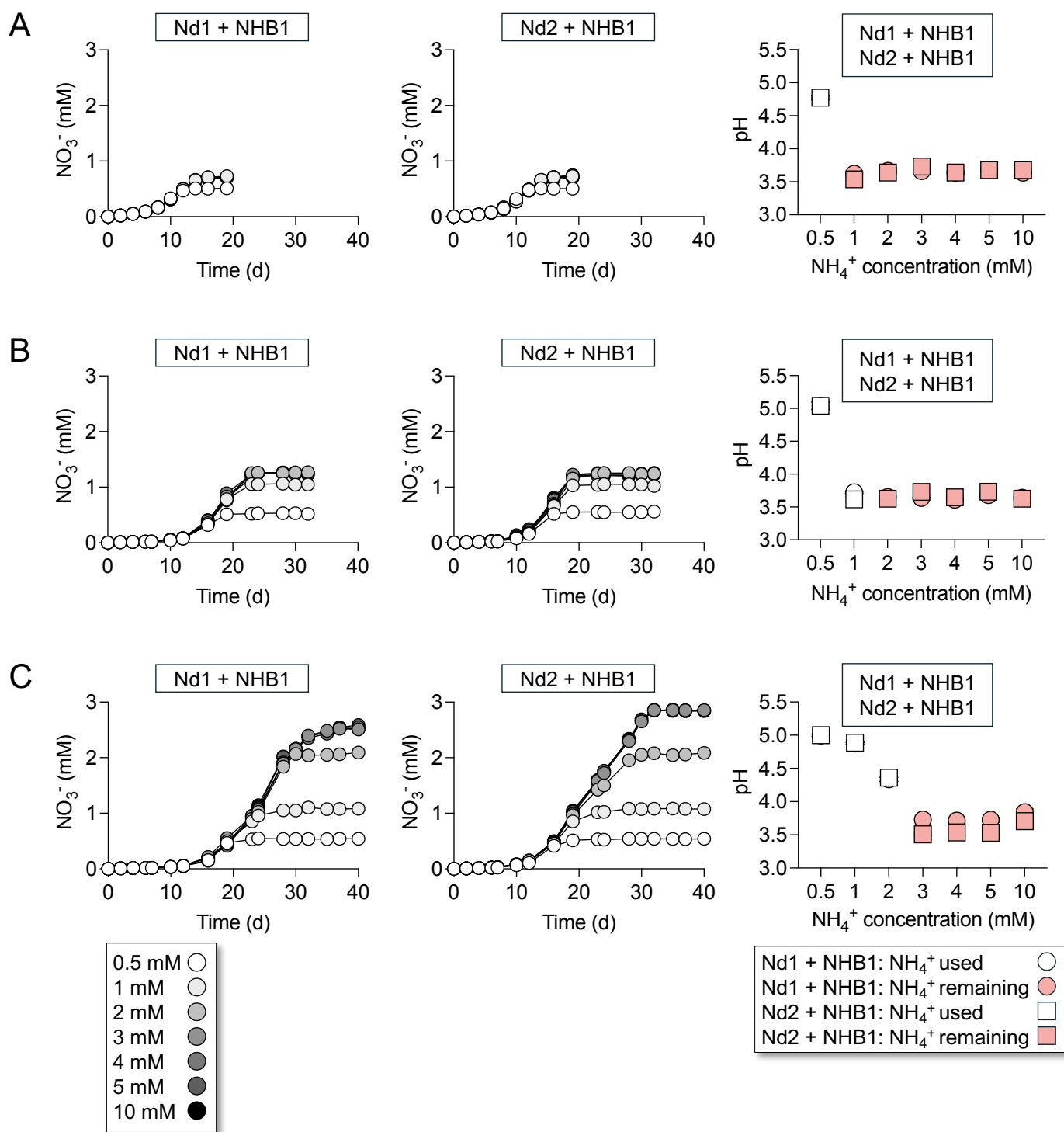


Figure S2. Effect of MES buffer concentration (**A** 10 mM, **B** 20 mM, **C** 50mM) on the growth of co-cultures of ‘*Ca. Nitrobacter laanbroekii*’ NHB1 and *Nitrosotalea devaniterrae* Nd1 or *Nitrosotalea sinensis* Nd2 in medium with an initial pH 5.2 and different NH₄⁺ concentrations (0.5, 1, 2, 3, 4, 5 and 10 mM). NO₃⁻ concentrations were determined through to stationary phase and pH measured at the last time point. The presence of remaining NH₄⁺ at the end of the stationary phase is described and demonstrates inhibition by decreasing pH rather than substrate limitation. Mean values and standard errors (mostly smaller than symbol size) from triplicate cultures are plotted in all panels.

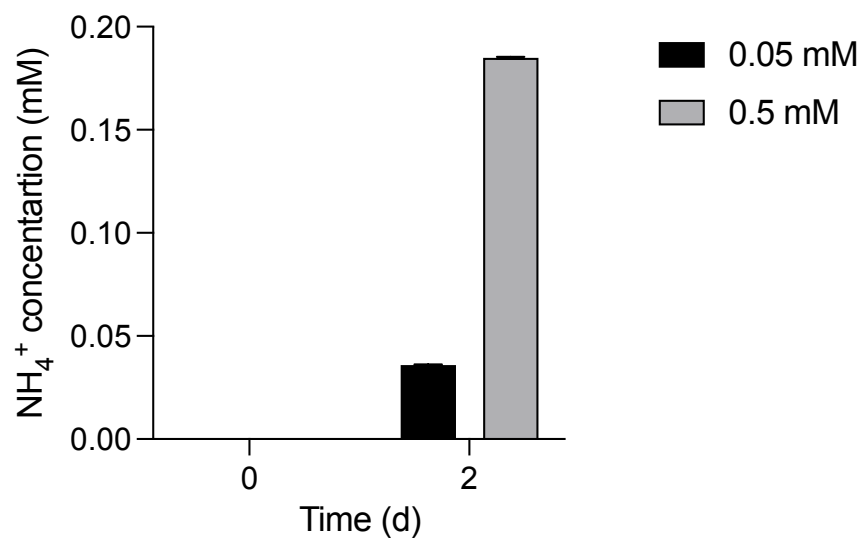


Figure S3. Production of NH_4^+ from abiotic decomposition of cyanate in pH 5.2 'freshwater medium' amended with 0.05 mM or 0.5 mM after two days incubation at 25°C.

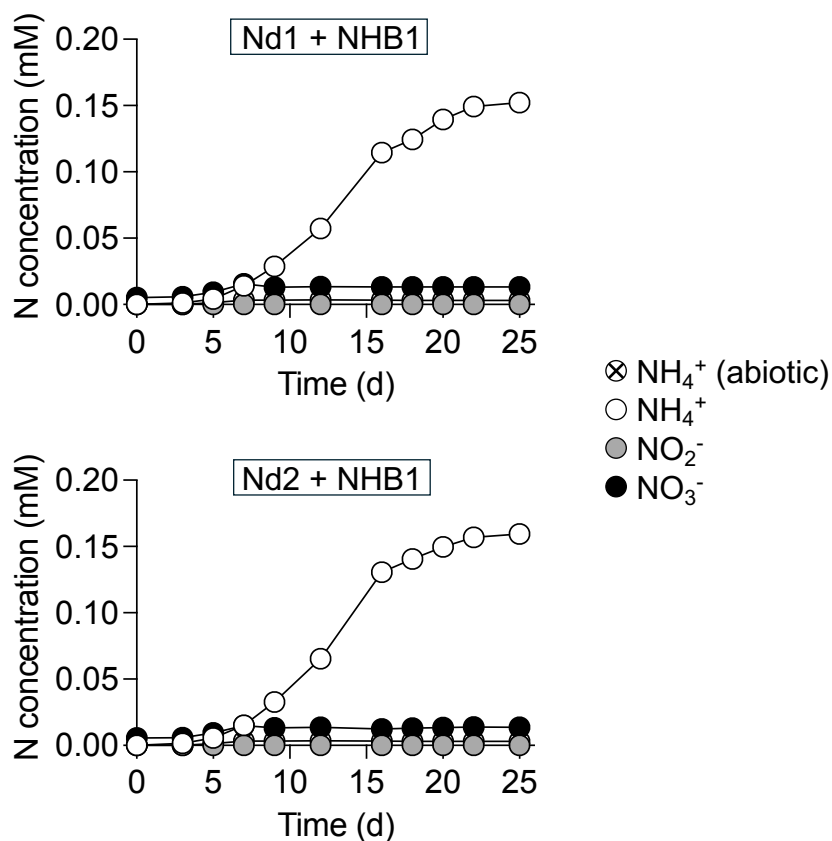


Figure S4. Inhibition of acidophilic AOA in ‘freshwater medium’ batch cultures (pH 6.0) amended with 0.5 mM cyanate. Cultures were inoculated with a previously established co-culture of ‘*Ca. Nitrobacter laanbroekii*’ NHB1 and *Nitrosotalea devaniterrae* Nd1 or *Nitrosotalea sinensis* Nd2. Less than 5% of NOB-generated ammonium was oxidised through to NO_3^- with no accumulation of NO_2^- . Decomposition of cyanate in the abiotic control produced $<3.5 \mu\text{M}$ NH_4^+ during the period of incubation. Mean values and standard errors (mostly smaller than symbol size) from triplicate cultures are plotted.

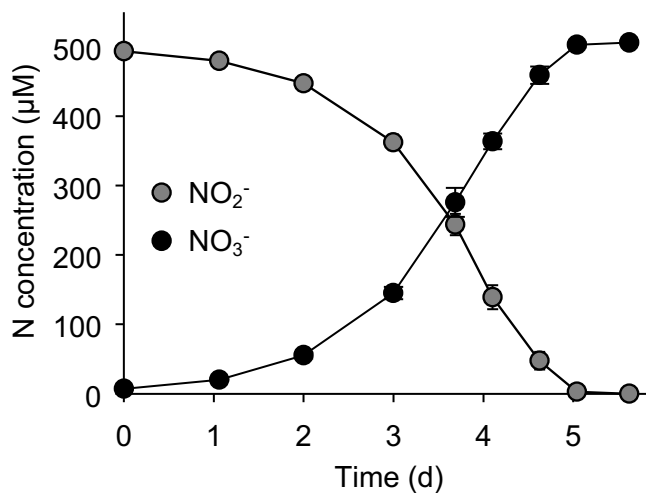


Figure S5. Stoichiometric relationship of NO_2^- consumption and NO_3^- production during growth of '*Ca. Nitrobacter laanbroekii*' NHB1. The medium was adjusted to pH 5.5 with an initial NO_2^- concentration of 500 μM and inoculated with 1% early stationary culture (vol/vol) and incubated at 25°C in the dark. Plotted values are the mean and standard errors of NO_2^- and NO_3^- concentrations of triplicate cultures. The confirmed stoichiometry between NO_2^- consumption and NO_3^- production enabled the use of NO_2^- concentrations to infer NO_3^- concentrations in subsequent culture characterisation experiments.

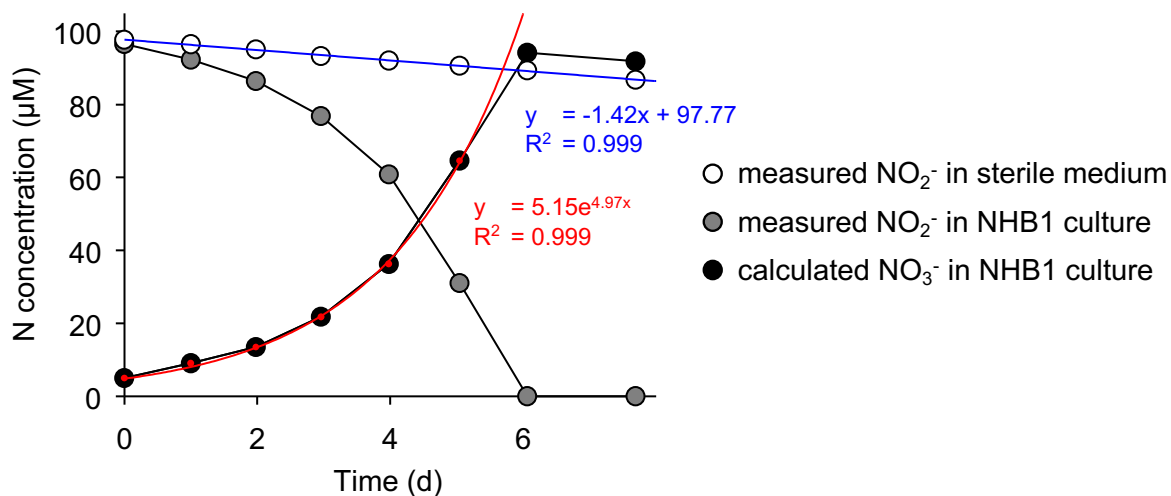


Figure S6. An example of assessing μ_{max} of '*Ca. Nitrobacter laanbroekii*' NHB1 when grown at pH 4.5 with an initial 100 μM NO_2^- concentration. Medium was inoculated with a 1% transfer (vol/vol) of an early stationary culture that had consumed 500 μM NO_2^- . Sterile medium served as control to monitor abiotic NO_2^- degradation following a linear decline over time (blue regression and equation). Measured NO_2^- concentrations in the growing culture and the abiotic degradation rate NO_2^- in the sterile control was used to calculate predicted NO_3^- concentrations. An exponential curve was then fitted to the NO_3^- data during exponential growth of the culture (red circles, regression and equation). The parameter in the exponent of the equation corresponds to the μ_{max} value of the culture.

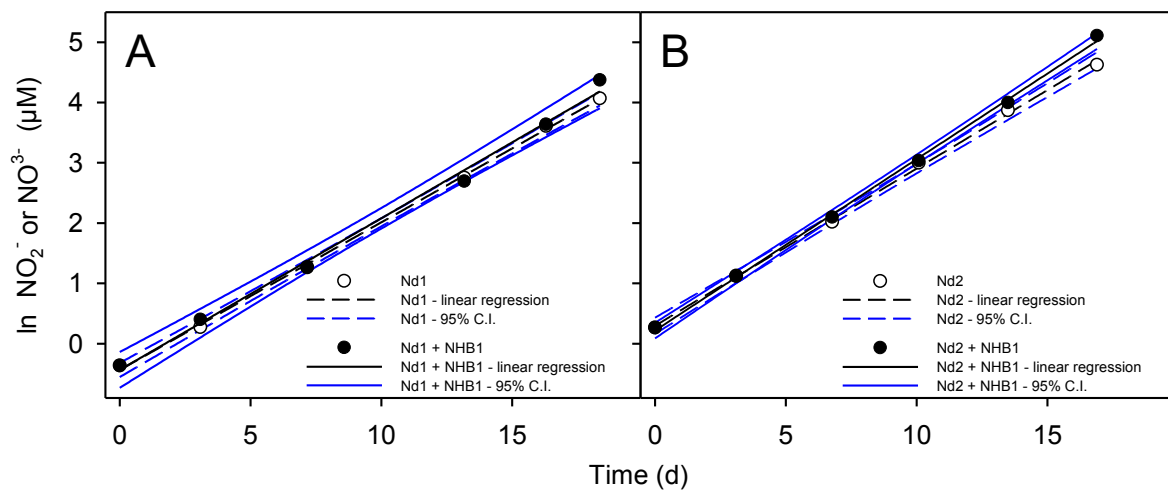


Figure S7. Examples of plots used for calculating maximum specific growth rates of *Nitrosotalea devaniterrae* Nd1 and *Nitrosotalea sinensis* Nd2 (assessed via NO₂⁻ production) or in co-culture with ‘*Ca. Nitrobacter laanbroekii*’ NHB1 (assessed via NO₃⁻ production) in medium supplied with 500 μM NH₄⁺. Linear regression and 95% confidence intervals (C.I.) were calculated from natural logarithm (ln) values of NO₂⁻ concentrations during the exponential phase of growth.