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Low yield and abiotic origin of N₂O formed by the complete nitrifier *Nitrospira inopinata*

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Nitrous oxide (N₂O) and nitric oxide (NO) are atmospheric trace gases that contribute to climate change and affect stratospheric and ground-level ozone concentrations. Ammonia oxidizing bacteria (AOB) and archaea (AOA) are key players in the nitrogen cycle and major producers of N₂O and NO globally. However, nothing is known about N₂O and NO production by the recently discovered and widely distributed complete ammonia oxidizers (comammox). Here, we show that the comammox bacterium *Nitrospira inopinata* is sensitive to inhibition by an NO scavenger, cannot denitrify to N₂O, and emits N₂O at levels that are comparable to AOA but much lower than AOB. Furthermore, we demonstrate that N₂O formed by *N. inopinata* formed under varying oxygen regimes originates from abiotic conversion of hydroxylamine. Our findings indicate that comammox microbes may produce less N₂O during nitrification than AOB.

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Nitrous oxide (N_2O) is the third most abundant greenhouse gas in the atmosphere. It contributes ~6% to the total radiative forcing and is also predicted as the dominant ozone depleting substance throughout the 21st century¹. The atmospheric N_2O concentration has continuously increased over the last decades at an average rate of ~0.31% per year, and this trend will continue^{1,2}. Anthropogenic emissions of N_2O make up 30–45% of the total global budget, with about two-thirds of this coming from agricultural and soil sources¹, and are predicted to increase in the future as application of nitrogen fertilizers rises to feed the growing human population^{1,3}. Microbial transformations of nitrogenous compounds, especially heterotrophic denitrification and chemolithoautotrophic aerobic nitrification, are the dominant contributor to N_2O emissions from agriculture and soil management as well as wastewater treatment, the latter of which adds ~3.4% to the global N_2O emission budget^{1,3}. In addition to N_2O , denitrifying and nitrifying microbes also release nitric oxide (NO) that represents an important metabolic intermediate for both guilds⁴. This activity is also environmentally relevant as NO contributes to the production of ground-level ozone and acid rain⁵. Thus, understanding the microbial players involved in NO/ N_2O production, the pathways that lead to the generation of these gases, and the environmental factors that control their fluxes is critical to modeling future emissions and developing appropriate mitigation strategies.

Classically, nitrification has been thought of as a two-step process. Ammonia (NH_3) is oxidized via hydroxylamine (NH_2OH) to nitrite (NO_2^-) by AOB and AOA, and subsequently nitrite oxidation to nitrate (NO_3^-) is catalyzed by nitrite oxidizing bacteria (NOB)⁴. Within the ammonia oxidizing microbes, two pathways were traditionally thought to contribute to NO and N_2O emissions: (1) aerobic N_2O formation from the abiotic reaction of the intermediate NH_2OH with NO_2^- (also referred to as hybrid N_2O formation), and (2) enzymatically catalyzed NO_2^- reduction to N_2O via NO through “nitrifier-denitrification”^{6,7}. The first pathway has been described for AOB and AOA^{8,9}, while the latter has only been reported for AOB^{8,10}. In AOB, hybrid N_2O formation is the dominant process at atmospheric oxygen levels, while nitrifier-denitrification is more important at low O_2 tension^{9,11–13}. Very recently, two additional routes for NO/ N_2O production from AOB have been characterized. Firstly, the periplasmic tetraheme cyt. *c* P460 protein (CytL, present in most but not all AOB) oxidizes two molecules of NH_2OH to N_2O and water under anoxic conditions¹⁴, although the kinetics of this protein may render it inefficient at this role under physiological conditions. Additionally, this protein can bind NO and reduce it

in the presence of NH_2OH to N_2O ¹⁴. Second, NO (and not NO_2^- as previously considered) is formed by the activity of hydroxylamine dehydrogenase (HAO) under oxic and anoxic conditions^{14,15}. CytL is used by AOB to detoxify NH_2OH and NO, while the activity of HAO leading to NO formation, which is further oxidized by an unknown enzyme to NO_2^- , is essential for energy conservation in these organisms (Fig. 1). Pure culture work on marine and soil AOA, and soil microcosm studies on complex communities, strongly suggest that archaea produce lower yields of N_2O than AOB ($\text{N}_2\text{O}/\text{NO}_2^-$ ratio %; 0.04–0.07% for AOA, 0.095–0.27% for AOB) during aerobic ammonia oxidation^{8,16–18}.

Two-step nitrification requires coupling between ammonia oxidation and nitrite oxidation. Consequently, the two steps can also become uncoupled, for example, under high nitrogen load, which can inhibit NOB^{19,20}. Additionally, mismatched nutrient affinities for NH_3 and NO_2^- between the two guilds and varying energetic constraints often allow for the accumulation of NO_2^- ^{20–22}. The accumulated NO_2^- from uncoupled nitrification can drive production of N_2O from hybrid formation, nitrifier-denitrification, and heterotrophic denitrification. In addition, it is important to keep in mind that NO and N_2O can also be produced by a multitude of chemical reactions that use the key metabolites of ammonia oxidizers – NH_2OH and NO_2^- (or its protonated form HNO_2) – as the main precursors (for recent reviews see ref. 5).

Recently, the traditional perspective that the two steps of nitrification are always catalyzed by different microorganisms was refuted by the discovery of ‘comammox’ organisms that oxidize ammonia to nitrate on their own^{23,24}. So far, comammox is restricted to the phylogenetic lineage II within the bacterial genus *Nitrospira*, which also contains canonical NOB²⁵. Comammox genomes contain all genes encoding the known bacterial machineries for ammonia and nitrite oxidation – ammonia monooxygenase (AMO), HAO, the hydroxylamine ubiquinone reduction module (HURM), and nitrite oxidoreductase (NXR)^{23,24,26,27}. Comammox *Nitrospira* are widely distributed and have been detected in various environments including pristine and agricultural soils, freshwater habitats, drinking water treatment systems, aquaculture biofilters, hot groundwater, and wastewater treatment plants in which they thrive, sometimes at considerable abundance^{23,24,27–31}.

Interestingly, a potential solution to the aforementioned synchrony problem between canonical AOB/AOA and NOB is naturally provided by the comammox organisms which perform ammonia oxidation and nitrite oxidation within one cell. Consistently, recent mathematical modeling has suggested that comammox organisms should show improved efficiency in nitrogen removal and a significantly reduced production of NO and N_2O ³². However, it is currently not known whether comammox bacteria actually produce NO and/or N_2O and if yes at which yields and via which pathways.

Recently, we obtained a pure culture of a comammox organism (*Nitrospira inopinata*) and characterized it kinetically³³. In this study, we use this pure culture to: (1) quantify NO and N_2O production by *N. inopinata*, (2) determine which biotic/abiotic pathways contribute to potential NO/ N_2O production in this organism, (3) compare the yields of NO/ N_2O from *N. inopinata* with known values for AOA and AOB, and (4) compare the genomic inventory for reactive nitrogen metabolism in various comammox organisms to gain insight into potential heterogeneity of their NO and N_2O production pathways. By using a combination of micro-respirometry, gas chromatography, NO scavenging assays, N_2O isotope analysis, and comparative genomics we demonstrate that *Nitrospira inopinata*, and possibly all other currently (meta)genomically characterized comammox

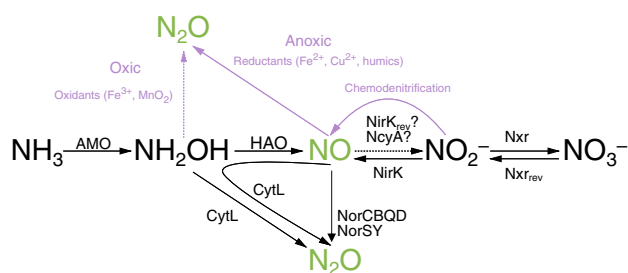


Fig. 1 Biotic and abiotic pathways leading to NO and N_2O production in bacterial nitrifiers. Black arrows depict confirmed (solid) and proposed (dashed) enzymatic reactions; confirmed or proposed enzymes are noted above or below the arrow. NcyA and NirK_{rev} are candidates for the NO-oxidizing (NOO) enzyme bacterial ammonia-oxidizers. Violet arrows represent abiotic reactions that occur under oxic (dashed) or anoxic (solid) conditions with violet text outlining the reactants/conditions that favor those processes

organisms, produce NO as an important intermediate but cannot denitrify to N₂O and thus produce low yields of N₂O that are comparable to soil AOA.

Results and discussion

Comammox *Nitrospira* lack NO reductases. NO and N₂O production by AOB, AOA, and NOB has been intensively studied and several key genes used by these nitrifiers for the production and consumption of these gases have been identified^{8,11,34–38} (Figs. 1 and 2). In contrast, no experimental data about the ability of comammox organisms to form NO and N₂O are available and nothing is known about the potential importance of these compounds for the metabolism of complete nitrifiers.

As a first step to close this knowledge gap, we updated previous comparative genomic analyses of comammox genomes^{26,27} and mined them for key genes that might be involved in NO and N₂O metabolism (Fig. 2). These analyses included *N. inopinata*, the only comammox organism available as pure culture³³, as well as genomes from two comammox enrichments and 15 comammox metagenome-assembled genomes (MAGs) from environmental samples retrieved via metagenomics^{23,26,29,30,39}. All comammox organisms encode ammonia oxidation machinery that is more closely related to AOB than to AOA²⁴. Part of this machinery is HAO that, in AOB, converts hydroxylamine (formed from NH₃ by the AMO in an oxygen-dependent manner) to NO via an O₂[–]-independent, three-electron oxidation step^{15,24}. Given its phylogenetic relationship to the HAO of AOB, and the fact that a planctomycete enzyme that shares multiple characteristics with HAO from *N. europaea* also produces NO⁴⁰, it seems highly likely that the HAO of comammox organisms also generates NO. Under this assumption, comammox organisms, like AOB, would benefit from the enzymatic oxidation of NO to NO₂[–] via an unknown NO oxidoreductase (NOO)⁴¹ in order to harvest a fourth electron for electron transport. For AOB the most compelling candidate for NOO is the red copper protein nitrosocyanin (NcyA), which is present in most AOB and is as highly expressed as AMO and HAO^{41,42}. However, none of the comammox *Nitrospira* genomes we analyzed encoded *ncyA*, suggesting an alternative candidate for NOO (Fig. 2). All of the genomes, with the exception of a single MAG (with an estimated completeness of 86.84%), contained the *nirK* gene. NirK is encoded by many (but not all) AOB, AOA, and NOB^{6,27,43–46} and catalyzes the one electron reduction of NO₂[–] to NO. NO generation by this enzyme is used by some AOB to facilitate efficient NH₃ oxidation and also under hypoxic conditions to enable intracellular redox-balance via nitrifier-denitrification (Supplementary Note 1)^{11,13,37,47}. Although NirK has been shown to operate reversibly and oxidize NO to NO₂[–]⁴⁸, the kinetics of the reaction are highly unfavorable at intracellular pH and redox potential, arguing that NirK is not an ideal candidate for the NOO.

In many AOB, the two-electron reduction of two molecules of NO to N₂O is performed by two classes of cytochrome *c* nitric oxide reductase (NOR) – *norCBQD* and the alternative NO reductase *norSY* – while all genome-sequenced AOA lack NOR^{10,27}. Physiological analyses of several AOB strains with or without cytochrome *c*-dependent NORs demonstrated that these enzymes are required for N₂O formation via nitrifier-denitrification^{6,37}. Interestingly, homologs for *norCBQD* and *norSY* are absent from all 23 currently known comammox MAGs and also absent from genomes of all cultivated strains including *N. inopinata* (Fig. 2)^{27,33}. Comammox bacteria might thus not be able to reduce NO to N₂O as part of the nitrifier-denitrification pathway under hypoxic conditions, similar to oligotrophic strains of AOB⁶.

We also queried the presence of the *c* cytochromes P460 (*cytL*) and *c*'-beta (*cytS*) in the genomes of comammox *Nitrospira*. The P460 enzyme from *N. europaea* is considered to be a detoxifying enzyme¹⁴ as it converts two equivalents of NH₂OH (or one NH₂OH and one NO) to N₂O under anoxic conditions, though this enzyme is expressed during aerobic growth in some but not all tested AOB^{14,42}. The function of CytS is still unknown, but for AOB it has been suggested to be involved in the oxidation/reduction of N-oxides or electron transfer for either detoxification or energy conservation⁴⁹. Both the cytochrome P460 and cytochrome *c*'-beta are found sporadically in genomes of comammox *Nitrospira* (Fig. 2). All of the currently enriched or cultured comammox representatives including *N. inopinata* lack *cytL*, while the genomes of *N. inopinata* and *Ca. N. nitrosa* contain the uncharacterized *cytS* (Fig. 2).

Taken together, *N. inopinata* and all other genome-sequenced comammox microbes possess the genetic potential to produce NO via HAO or NirK activity, but lack *bona fide* NO reductases to form N₂O. However, keeping in mind that ~46% of the *N. inopinata* genes have no functional annotation and several, often unrelated, enzyme classes can catalyze identical transformations of nitrogen compounds⁵⁰, physiological experiments as well as protein purification and characterization are clearly required to examine formation, magnitude, and importance of NO and N₂O formation and NO oxidation in comammox organisms.

N. inopinata releases and consumes NO under oxic conditions.

To determine whether *N. inopinata* produces and consumes NO, instantaneous O₂ and NO kinetics were measured with micro-sensors during NH₃ and NO₂[–] oxidation by *N. inopinata*. Addition of 250 μM NH₄⁺ into the micro-respirometry (MR) chamber led to immediate substrate-dependent O₂ consumption and NO production. NO production peaked at ~13 nM after ~30% of the dissolved O₂ was consumed, followed by net NO consumption (Fig. 3). Since *N. inopinata* is an oligotroph, we also tested how substrate concentration influences net NO flux. Lower substrate concentrations (<15 μM NH₄⁺) resulted in significantly less NO production (<0.8 nM) (Supplementary Fig. 1). There was no measurable NO production after O₂ was depleted in the presence of NH₃, reflecting the dependency of NO production on O₂ and strongly suggesting that *N. inopinata* under the conditions applied did not respire anaerobically with NO₂[–] as an electron acceptor using storage products as electron donors. Despite comparable absolute cell numbers (~1 × 10¹⁰ cells), net production of NO (30 nM per ~1 × 10¹⁰ cells) by the *N. inopinata* biomass was about an order of magnitude lower during aerobic oxidation of the same amount of NH₃ than by the oligotrophic AOB *Nitrosomonas* sp. Is79A3 or *N. ureae*⁶. Furthermore, previous work demonstrated that both of these oligotrophic AOB produce very large (>250 nM) quantities of NO after the onset of hypoxia (ascribed to the activity of their NirK enzymes). This phenomenon reflects the absence of NO reductases in these strains, which are required for NO reduction to N₂O in the nitrifier-denitrification pathway^{6,37}. In contrast, the NO reductase encoding *Nitrosomonas europaea* ATCC 19718 produced ~180–210 nM NO (per ~1 × 10¹⁰ cells) throughout aerobic NH₃ oxidation and consumed NO via nitrifier denitrification after the onset of hypoxia (Supplementary Fig. 2). Consistently, other previously analyzed AOB also produced >50 nM NO (per 1 × 10¹⁰ total cells) during aerobic NH₃ oxidation prior to hypoxia in the MR chamber⁶.

The lack of measurable NO production by *N. inopinata* during O₂-limited conditions despite the fact that NirK was the second most abundant protein in proteomic analysis of *N. inopinata* even under aerobic conditions (Supplementary Fig. 3) suggests that

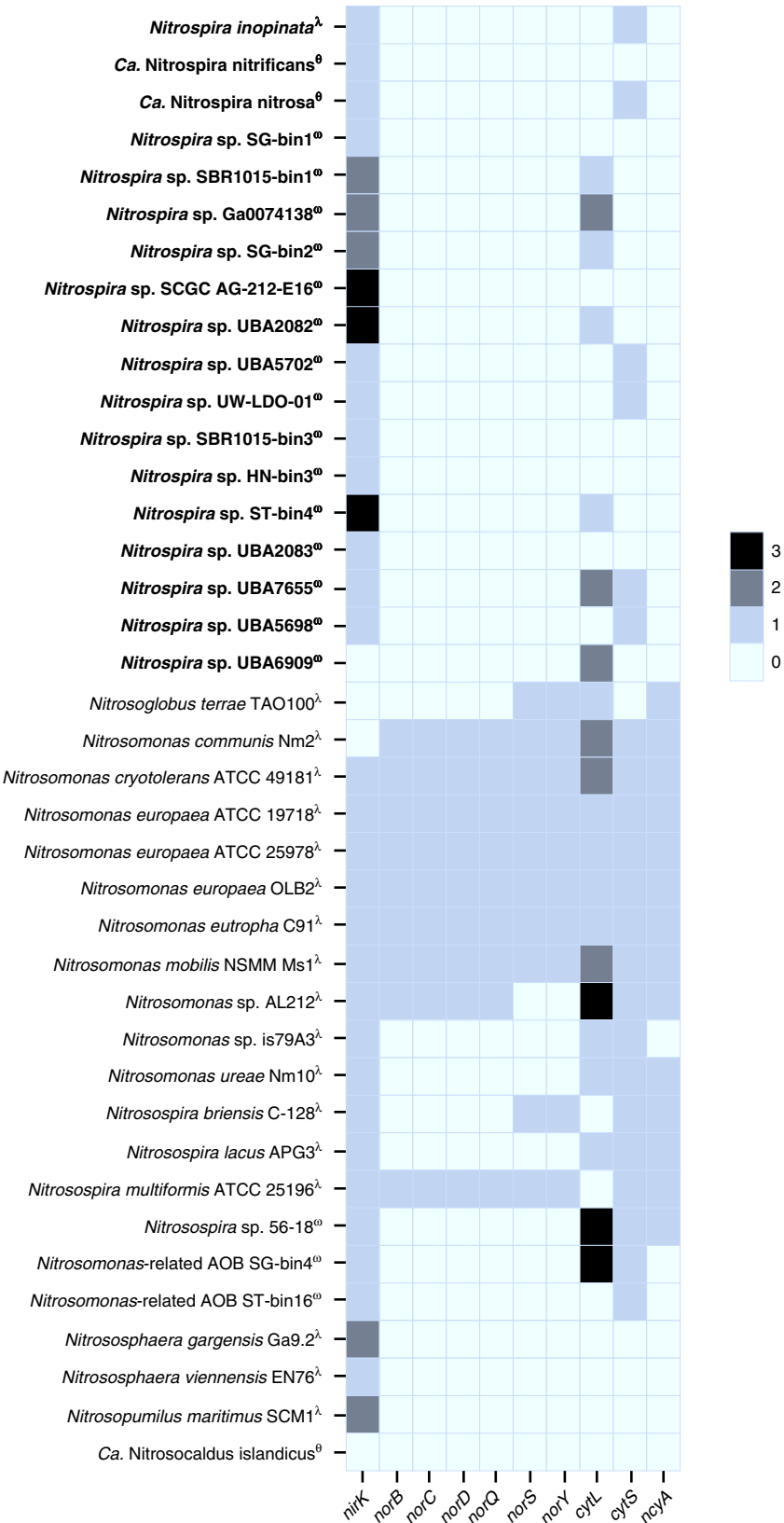


Fig. 2 Gene inventory implicated in NO_x metabolism in publicly available genomes of various nitrifiers. Comammox *Nitrospira* genomes are depicted in boldface. Genomes from enrichments and pure cultures are denoted by the symbols θ and λ , respectively. The symbol ω denotes uncultured organisms. The number of copies of each gene are denoted by the color bar. Published genomes and MAGs were compiled from the literature (see Supplementary Data 1 for sources, source data, locus tags, and bin/genome statistics) and downloaded from the Integrated Microbial Genomes website (<https://img.jgi.doe.gov/cgi-bin/mer/main.cgi>)

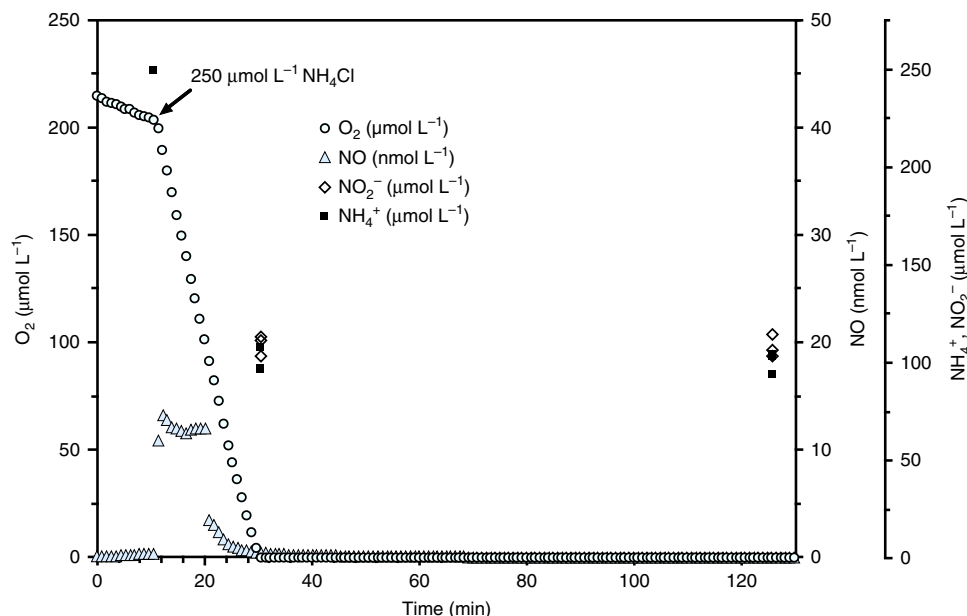


Fig. 3 Instantaneous O_2 consumption and NO production during NH_3 oxidation by *N. inopinata*. The data shown here is a single representative of three biological replicates ($n = 3$). Two additional biological replicates are shown in Supplementary Fig. 6. Dissolved O_2 is shown in open circles, dissolved NO in filled gray triangles, NO_2^- in open diamonds, and NH_4^+ in filled squares. The NH_4^+ concentration immediately after injection (~ 10 min) was inferred from the injected volume of a stock NH_4Cl solution, otherwise NO_2^- and NH_4^+ concentrations were determined in three technical replicates ($n = 3$). Experiments were performed in a microrespiration (MR) chamber fitted with O_2 and NO microsensors. The arrow marks the addition of $250 \mu M NH_4Cl$ into the MR chamber. About $110 \mu M$ residual NO_2^- was present in the chamber once O_2 reached a concentration below the detectable level at ~ 35 min. No NO formation from NH_4^+ was measurable in sterile media controls containing the same amount of heat-killed biomass of *N. inopinata*. Source data are provided as a Source Data file

NirK either has very weak activity under hypoxic conditions, producing NO below the detection limit of our instrument (~ 0.25 nM NO), or that *N. inopinata* does not perform NirK-based nitrifier-denitrification during hypoxia at all. The NO profile during aerobic NH_3 oxidation by *N. inopinata* shows interesting similarities to that previously reported for the AOA strain *N. viennensis*, which also rapidly produces NO and then consumes it during aerobic NH_3 oxidation¹⁰. However, in contrast to *N. inopinata*, *N. viennensis* additionally produces NO at the onset of hypoxia¹⁰. Although the NO production and consumption profiles of *N. inopinata* and the AOA strains *N. viennensis* and *N. maritimus* are different^{10,51,52}, NirK is strongly expressed in all three organisms^{53,54} and the release of NO appears to be more tightly controlled by them than in AOB.

Interestingly, using NO_2^- instead of NH_3 as the electron donor resulted in a different NO production and consumption profile by *N. inopinata* (Fig. 4); addition of 2.5 mM NO_2^- led to immediate net NO production after which NO levels reached a steady-state level of ~ 45 nM (Fig. 4). Net consumption of NO was not evident during further NO_2^- oxidation. In contrast, instantaneous NO concentrations were below our detection limit (~ 0.25 nM) for the closely related non-comammox *Nitrospira*, *N. moscoviensis*, during NO_2^- oxidation (Supplementary Fig. 4). To our knowledge, NO production in *Nitrospira* has not been investigated previously but previous work has shown that NO (65 nM NO in the liquid phase) inhibits NO_2^- oxidation to NO_3^- in *Nitrospira* dominated sludge⁵⁵. The more distantly related proteobacterial nitrite-oxidizer, *Nitrobacter winogradskyi* produces³⁸ and consumes NO ^{36,38} in a NO_2^- -dependent manner during normal aerobic growth. However, the total NO flux is strongly influenced by its quorum sensing system and decreased when quorum sensing was quenched³⁸.

It is interesting to note that in *N. winogradskyi* the NO producing nitrite reductase NirK has been postulated to facilitate

nitrite oxidation under O_2 -limiting conditions by maintaining redox balance via regulation of electron flow. At low oxygen tension in the presence of nitrite, NirK is strongly upregulated and more NO is produced causing reversible inhibition of the heme-copper cyt *c* terminal oxidase and thus intensifying reverse electron transport for $NAD(P)^+$ reduction and storage compound formation³⁶. In contrast, we show here that stable NO production by *N. inopinata* during NO_2^- oxidation occurs under fully oxic conditions, which is consistent with a constitutive high expression of NirK during aerobic growth of *N. inopinata* (Fig. 4) (Supplementary Fig. 3). Alternatively, it is conceivable that the contrasting NO profiles in *N. moscoviensis* and *N. inopinata* are caused by a reversal of the yet unknown NOO enzyme in *N. inopinata* at very high NO_2^- concentrations, a process that does not occur in *N. moscoviensis* because it lacks the genetic repertoire to oxidize NH_3 to NO_2^- .

PTIO is a potent inhibitor of NH_3 oxidation in *N. inopinata*.

Both AOB and AOA produce NO as an obligate intermediate during NH_3 oxidation^{10,41,51,52}. However, AOA exhibit very tight control over the production and consumption of NO during NH_3 oxidation^{10,51,52}. This difference in the regulation of NO concentrations may explain why the AOA are selectively inhibited by low concentrations of the NO scavenger PTIO^{51,56}. The similar aerobic NO kinetics between *N. inopinata* and the AOA *N. viennensis* raised the hypothesis that comammox *Nitrospira* may be sensitive to inhibition by low concentrations of PTIO as well. We tested this hypothesis by comparing instantaneous O_2 consumption rates and batch culture NH_3 oxidation activity of *N. inopinata* in the presence of various concentrations of PTIO to non PTIO-amended biomass (Supplementary Fig. 5). Indeed, PTIO was a potent inhibitor of instantaneous O_2 consumption and batch culture NH_3 oxidation in *N. inopinata* with a half-

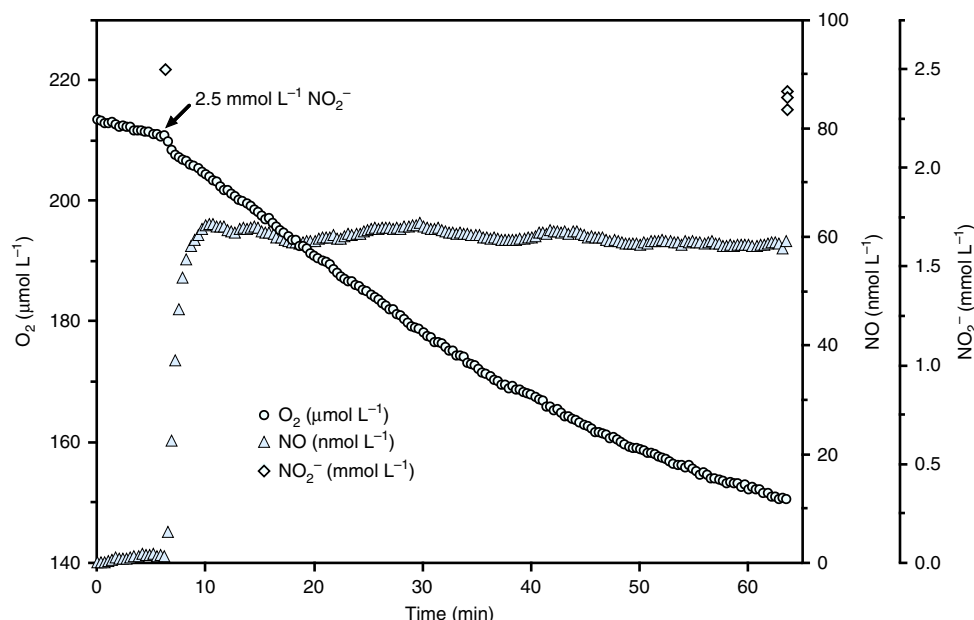


Fig. 4 Instantaneous O_2 consumption and NO production during NO_2^- oxidation by *N. inopinata*. The data shown here represents a single replicate of three biological replicates ($n = 3$). Two additional biological replicates are shown in Supplementary Fig. 8. Dissolved O_2 is shown in open circles, dissolved NO in filled gray triangles, and NO_2^- in open diamonds. The NO_2^- concentration immediately after injection (~6 min) were inferred from the injected volume of a stock $NaNO_2$ solution, otherwise NO_2^- concentrations were determined in three technical replicates ($n = 3$). Experiments were performed in a 10-mL microrespiration (MR) chamber fitted with an O_2 and NO microsensors. The arrow marks the addition of 2.5 mM NO_2^- into the MR chamber. No NO formation from NO_2^- was measurable in oxic sterile media controls containing heat-killed biomass. Source data are provided as a Source Data file

effective maximal concentrations (EC_{50}) of 18.9 and 63.6 μM , respectively. The EC_{50} of PTIO for *N. maritimus* and *N. viennensis* ranges from 17.5–18.3 μM , while *N. multiformis* and other AOB are only inhibited by PTIO concentrations $>300 \mu M$ ^{51,56}. Washing and reharvesting *N. inopinata* cells treated with low but inhibitory concentrations of PTIO (33–100 μM PTIO) to remove the PTIO resulted in complete recovery ($>95\%$) of activity, while cells treated with high concentrations of PTIO only showed partial (mean $\pm$ standard deviation: $28.3 \pm 11.9\%$, 330 μM PTIO) or no recovery of activity ($1.8 \pm 3.7\%$, 1000 μM PTIO; Supplementary Fig. 5). These results are consistent with PTIO acting as a NO-binding, reversible inhibitor at low concentrations, and a irreversible cytotoxin at high concentrations. However, it still remains a possibility that the PTIO-dependent inhibition we observed was caused by the imino nitroxides (PTIs) and NO_2 formed by the reaction of PTIO and NO rather than NO chelation.

To determine whether PTIO exhibits general cytotoxicity toward *Nitrospira* cells, we tested the effect of PTIO on instantaneous O_2 consumption and growth in the non-ammonia oxidizing *N. moscoviensis*. Generally, PTIO was significantly less inhibitory to *N. moscoviensis*, with estimated EC_{50} values of 385.6 μM and 100.2 μM for instantaneous activity and batch culture NO_2^- oxidation, respectively (Supplementary Fig. 5). However, considering that PTIO was not fully inhibitory to *N. moscoviensis* at even 1000 μM PTIO during batch culture growth, the calculated EC_{50} in this condition is not reliable. Nearly complete inhibition ($<10\%$ activity) was only evident when we measured instantaneous O_2 consumption in the 1000 μM PTIO treatment and the calculated EC_{50} under these conditions (385.6 μM) is very similar to that of previously published values for AOB⁵¹. Finally, washing and reharvesting *N. moscoviensis* cells that were significantly inhibited by PTIO resulted in no measurable recovery when compared to an untreated control ($6.3 \pm 3.3\%$, 1000 μM PTIO + wash; Supplementary Fig. 5). Collectively, the inhibitory effect of PTIO on *N.*

moscoviensis at these concentrations is permanent even after it is removed and this suggests that PTIO at high concentrations acts as a irreversible cytotoxin in *N. moscoviensis*.

Taken together, the NO production profile, the low EC_{50} of PTIO, and the reversibility of PTIO toxicity in *N. inopinata* suggest an essential role for NO as an intermediate in *N. inopinata*. Furthermore, our data reveal that PTIO can no longer be considered as a selective inhibitor of AOA in studies targeted at assessing the roles of various nitrifying microbes in environmental systems as comammox *Nitrospira* will also be inhibited upon addition of low concentrations of this NO scavenger. Furthermore, inhibition of other nitrifying microbes not mediated by NO scavenging but by the potential cytotoxicity of PTIO, especially at higher concentrations, has to be considered in the design of such molecular ecology experiments.

Respirometry suggests abiotic N_2O formation by *N. inopinata*.

AOB and AOA release small amounts of NH_2OH during ammonia oxidation under fully oxic conditions and the abiotic conversion of NH_2OH with Fe^{3+} , Mn^{4+} , Cu^{2+} , NO_2^- , and other components of the surrounding matrix under oxic conditions⁵⁷ explains most of the N_2O formation by these nitrifiers under these conditions^{6,7,10}. Hypoxia leads to a significant increase in the N_2O yield from NH_3 in AOB^{9,12,13,58}, while oxygen concentration has no major influence on the N_2O yields of all tested AOA^{8,17,52}. Main pathways that contribute to N_2O formation during O_2 limitation in these nitrifiers are nitrifier-denitrification –the sequential enzymatic reduction of NO_2^- to N_2O via NIR and NOR – and chemodenitrification, whereby NO_2^- or NO is non-enzymatically reduced to N_2O via media components or heat-killed cell moieties^{6,59}. The NO can be derived from enzymatic NO_2^- reduction or from reactions of NO_x with NH_2OH . Studies on AOB from diverse environments and adapted to different substrate concentrations showed that all of them produce N_2O within minutes of the transition from oxic to anoxic

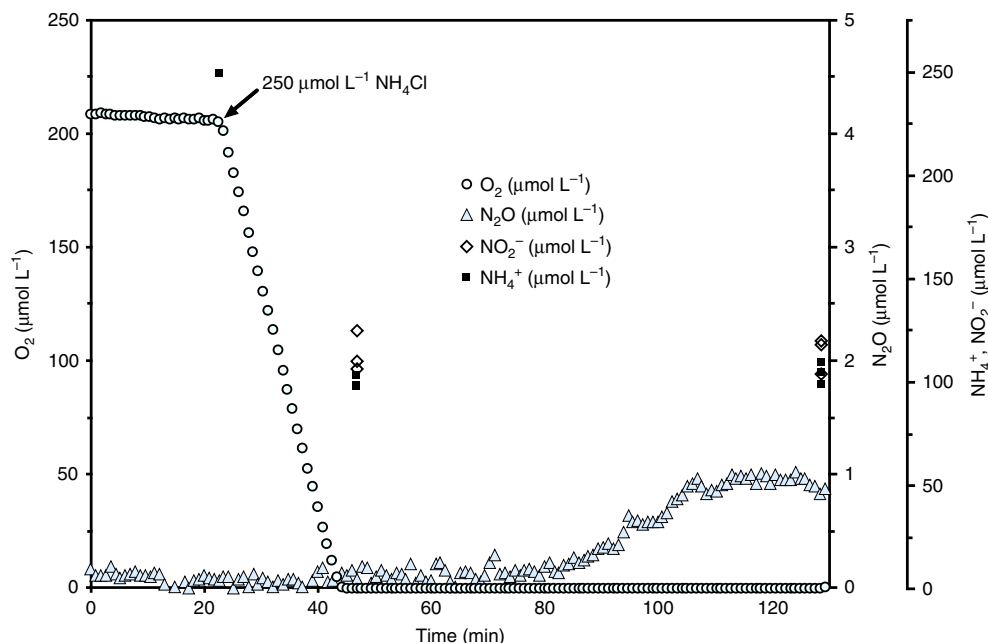


Fig. 5 Instantaneous O_2 consumption and N_2O production during NH_3 oxidation by *N. inopinata*. The data shown here is a single representative of three biological replicates ($n = 3$). Two additional biological replicates are shown in Supplementary Fig. 9. Dissolved O_2 is shown in open circles, dissolved N_2O in filled gray triangles, NO_2^- in open diamonds, and NH_4^+ in filled squares. The NH_4^+ concentration immediately after injection (~ 23 min) was inferred from the injected volume of a stock NH_4Cl solution, otherwise NO_2^- and NH_4^+ concentrations were determined in three technical replicates ($n = 3$). Experiments were performed in a 10-mL microrespiration (MR) chamber fitted with O_2 and N_2O microsenors. The arrow marks the addition of $250 \mu M$ NH_4Cl into the MR chamber. About $110 \mu M$ residual NO_2^- was present in the chamber once O_2 reached below the detectable level at ~ 45 min. Source data are provided as a Source Data file

conditions⁶. This released N_2O originates mainly from nitrifier-denitrification in AOB that encode nitric oxide reductases (*norCBQD* or *norSY*) and to a much lower extent from chemo-denitrification in oligotrophic AOB that lack NO reductases and emit large quantities of NO during transition from oxic to anoxic conditions⁶. The soil thaumarchaeon *N. viennensis*, which cannot perform nitrifier-denitrification to N_2O , also releases large quantities of NO during hypoxic conditions which then reacts abiotically with medium components (Cu^{2+} and Fe^{2+}) to form N_2O ^{8,10}.

Using NH_3 as the electron donor, we measured instantaneous N_2O production with microsenors by *N. inopinata* biomass during oxic conditions and through a period of hypoxia. Interestingly, *N. inopinata* did not produce N_2O during aerobic NH_3 oxidation or during the transition from oxic to anoxic conditions (Fig. 5) that could be measured with the N_2O microsenor that has a relatively low sensitivity (100 nM). Small quantities of N_2O were only measurable with the sensor from *N. inopinata* ~ 40 min after O_2 was depleted below the limit of detection (~ 300 nM dissolved O_2). This delayed N_2O accumulation did not coincide with NO release in replicate traces where NO was measured (Figs. 3 and S6). Unlike growth assays, these short micro-respirometry experiments with high biomass and high substrate concentrations likely result in a relatively large accumulation in intracellular reductant in the form of NH_2OH ⁵². To test whether this delayed N_2O release could originate from cell lysis and abiotic formation of N_2O from NO_2^- , NH_2OH , and other media components, we incubated heat-killed *N. inopinata* cells under anoxic conditions in AFW media supplemented with $1.8 \mu M$ NH_2OH and $100 \mu M$ NO_2^- . This NH_2OH concentration was chosen based on determined extracellular NH_2OH concentrations in enrichment cultures of *N. inopinata*⁷. These abiotic controls yielded $0.83 \pm 0.1 \mu M$ N_2O (mean $\pm$ standard deviation), explaining most of the observed N_2O in the live cell incubations.

Together, these results suggest that the delayed N_2O formation under O_2 -limiting conditions by *N. inopinata* originates mainly from abiotic reactions of accumulated NH_2OH and not from the enzymatic reduction of NO to N_2O .

***N. inopinata* N_2O originates from abiotic NH_2OH conversion.**

The short duration of the micro-respirometry experiments and the limited sensitivity of the N_2O microsenors (100 nM dissolved N_2O) makes calculating the precise N_2O yield during NH_3 oxidation very difficult. To calculate the precise yields of N_2O (as a N_2O/NH_3 ratio in percent) we performed batch growth experiments with *N. inopinata* biomass in sealed serum vials under NH_3 - and O_2 -limiting conditions and measured consumption of relevant metabolites and production of N_2O using gas chromatography. N_2O formation during oxic ($\sim 20.8\%$ O_2), NH_3 -limited growth was entirely dependent on NH_3 oxidation; no N_2O formation was observed during NO_2^- oxidation to NO_3^- after all of the NH_3 was depleted (Fig. 6a). Similarly, NO_2^- oxidation by *N. moscoviensis* in batch growth experiments yielded only trace (mean $\pm$ standard deviation: 4.3 ± 2.3 nM) amounts of N_2O under fully oxic and hypoxic conditions (Supplementary Fig. 7).

The N_2O yield (as a N_2O per NH_3 ratio %) during aerobic NH_3 oxidation for *N. inopinata* was 0.070 ± 0.006 (mean $\pm$ standard deviation). This measured N_2O yield closely matched the predicted yield of N_2O from abiotic reactions between media components and extracellular NH_2OH for *N. inopinata* cultivated at $500 \mu M$ NH_4^+ (0.06%)⁷. This suggests that all of the N_2O produced by *N. inopinata* during oxic growth on NH_3 came from the abiotic conversion of NH_2OH . Further, an N_2O yield of $0.073 \pm 0.033\%$ in O_2^- limited incubations that were initiated at $\sim 1.8 \mu M$ dissolved O_2 (equivalent to 0.89% O_2) demonstrated that the N_2O yield was not influenced by hypoxia (Fig. 6b). This matches observations in *N. maritimus* and *N. viennensis* in which the N_2O yield during growth

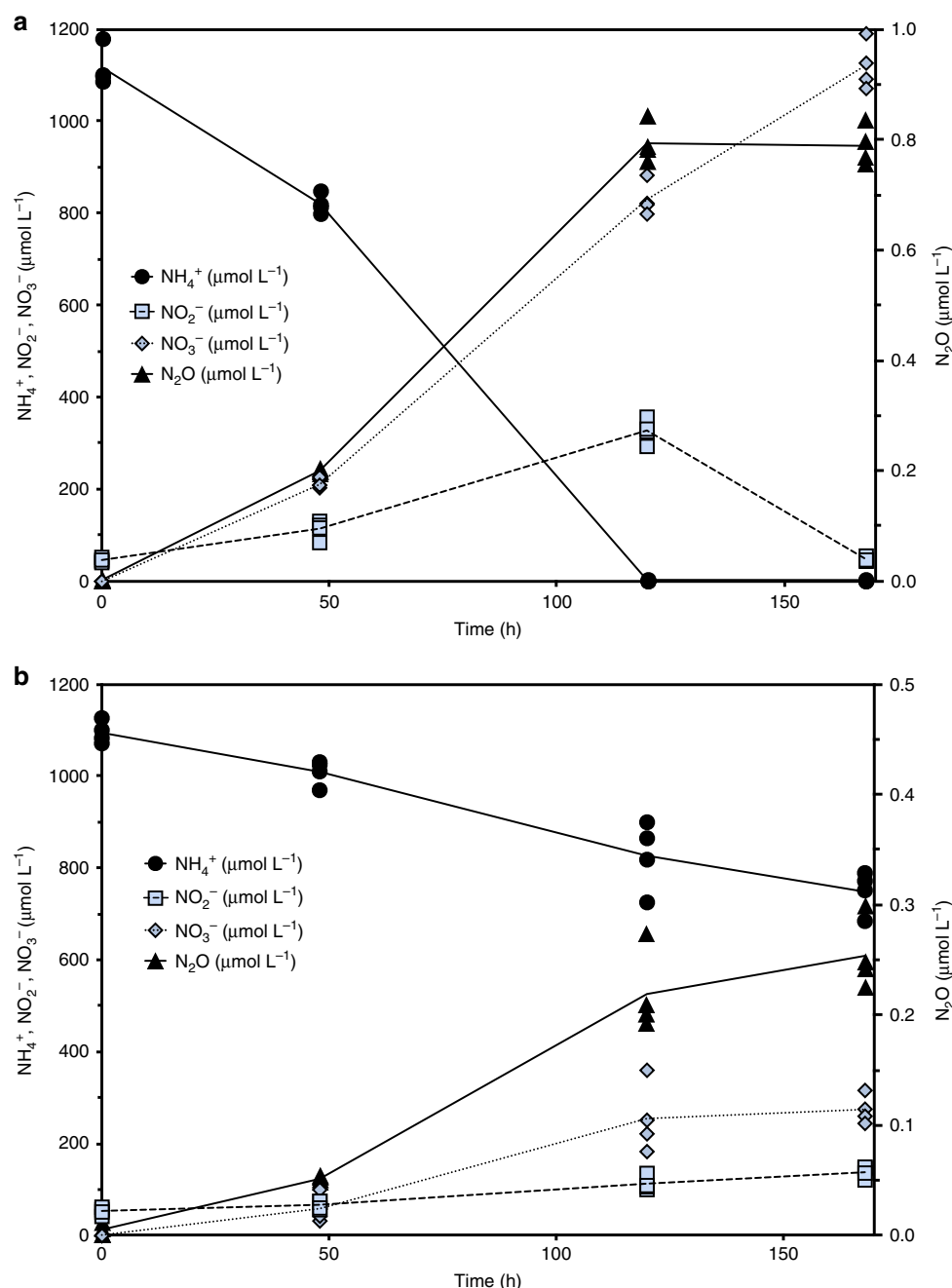


Fig. 6 Oxidation of NH_3 to NO_2^- and NO_3^- and concurrent N_2O yield during growth of *N. inopinata*. *N. inopinata* was incubated in closed serum vials under fully oxic conditions (initial O_2 at ~20.8%, **a**) or hypoxic conditions (initial O_2 at ~0.89%, **b**). NH_4^+ is shown in filled circles, NO_2^- in filled squares, NO_3^- in filled diamonds, and N_2O in filled triangles; for each time point, individual replicates ($n = 4$ biological replicates) are plotted and the means connected by a solid black line. The amount of biomass at $t = 0$ was the same in the oxic and hypoxic vials. NH_3 oxidation to NO_3^- is nearly stoichiometric in panel **a** due to unlimited O_2 ; in panel **b**, however, limiting O_2 concentrations prevented the stoichiometric oxidation of NH_3 to NO_3^- . O_2 concentrations in the hypoxic treatment at 168 h were below the limit of detection (300 nM). Source data are provided as a Source Data file

is not influenced by varying O_2 concentrations and is in stark contrast to AOB like *N. europaea* and *N. multiformis* that produce ~3 times more N_2O on a per mol NH_3 basis during nitrifier-denitrification under low O_2 conditions^{8,9,13,58}. Furthermore, the N_2O yield values for *N. inopinata* ($0.070 \pm 0.006\%$) are very similar to those observed for *N. viennensis* (0.07 to 0.09%) and other several other soil AOA (~0.080%)⁸.

To obtain additional information on the pathway(s) leading to N_2O production in *N. inopinata*, we determined the intramolecular (natural abundance) distribution of ^{15}N within the linear

N_2O molecule ($\text{N}^\beta\text{-N}^\alpha\text{-O}$) between the central (α) and the external (β) position (also called the site preference SP; $\delta^{15}\text{N}\text{-SP}$, defined as $\delta^{15}\text{N}^\alpha - \delta^{15}\text{N}^\beta$) from headspace N_2O harvested from batch yield experiments performed under NH_3 - and O_2 -limiting conditions (Fig. 6). The site preference of N_2O produced during NH_3 -limited growth (mean $\pm$ standard deviation: $33.4 \pm 0.3\text{‰}$) was the same as during hypoxia ($34.2 \pm 1.4\text{‰}$). The unvarying SP of N_2O produced during the different conditions reflects that the N_2O source did not change in response to hypoxia. Such a positive $\delta^{15}\text{N}\text{-SP}$ is also consistent with a N_2O source from

NH₂OH conversion (observed under oxic conditions to be 30.8–35.6%^{60,61}, for AOB and 13.1–30.8% for AOA^{16,62}), whereas N₂O produced via nitrifier-denitrification or heterotrophic denitrification has a SP near or below 0^{61,63,64}.

In summary, we first show that *N. inopinata* has very tight control over NO production during aerobic NH₃ oxidation, that it also produces NO during aerobic NO₂[−] oxidation, but does not release NO during hypoxia. These data collectively strongly suggest an important but not yet fully explored metabolic role of NO for complete nitrifiers of the genus *Nitrospira*. Consistently, low concentrations of the NO scavenger PTIO inhibit *N. inopinata*. Importantly, we then demonstrate that *N. inopinata* cannot denitrify to N₂O due to the absence of NO reductases in the genome, that it has a low N₂O yield that is comparable to cultivated soil AOA and not influenced by O₂ levels, and that the N₂O formed originates from abiotic NH₂OH conversion. While we cannot rule out the possibility that some comammox *Nitrospira* encode a currently unknown NO reductase, we hypothesize that all other currently enriched and/or metagenomically characterized comammox *Nitrospira* also lack the ability to denitrify to N₂O because they lack homologs for known NO reductases. Consequently, adoption of conditions that favor the growth of complete nitrifiers over AOB, for example, in engineered systems and in soils where comammox bacteria have been identified^{28,30,65}, may influence nitrification-dependent N₂O emissions. Quantifying the relative contribution of comammox organisms as well as canonical nitrifiers to ammonia oxidation and N₂O production in natural and engineered environments will thus be an important aim for follow-up research.

Methods

Strains and cultivation. *Nitrospira inopinata* was routinely cultivated at 37 °C in AOM medium²⁴ which contained (per L): 50 mg KH₂PO₄, 75 mg KCl, 50 mg MgSO₄ 7H₂O, 584 mg NaCl, 1 mL specific trace element solution (TES), and 1 mL of selenium-tungstate solution (SWS). TES contained (per litre of deionized water): 34.4 mg MnSO₄ × H₂O, 50 mg H₃BO₃, 70 mg ZnCl₂, 72.6 mg Na₂MoO₄ × 2H₂O, 20 mg CuCl₂ × 2H₂O, 24 mg NiCl₂ × 6H₂O, 80 mg CoCl₂ × 6H₂O, and 1 g of FeSO₄ × 7H₂O (dissolved in 2.5 mL 37% HCl)²⁴. SWS contained (per litre): 0.5 NaOH, 3 mg Na₂SeO₃ × 5H₂O, and 4 mg Na₂WO₄ × 2H₂O²⁴. The AOM medium was supplemented with 1 mM NH₄⁺ and 4 g L^{−1} of CaCO₃, the latter of which acts as a solid buffering system and substrate for attachment. The final pH of the medium was ~8.0 after sterilization. AOM medium could not be used to cultivate *N. inopinata* for the micro-respirometry experiments because the large quantities of undissolved CaCO₃ interfered mechanically with the highly sensitive NO sensor that, unlike the O₂ and N₂O sensors, has an extremely small signal (10^{−14} ampere). Consequently, Artificial Fresh Water Medium (AFW) was used to prepare *N. inopinata* biomass for the micro-respirometry (MR) experiments. The basal AFW (without pyruvate) containing 1 mM NH₄⁺ was supplemented with (per L): 1 mL of 1000X non-chelated trace element solution, 1 mL of 1000X vitamin solution, and 1 mL of 7.5 mM Fe-NaEDTA solution¹⁶. The final pH of the AFW was set to ~7.2 through the addition of HEPES (prepared initially to pH 7.6) and NaHCO₃, which were added at final concentrations of 4 mM and 3 mM, respectively. The pure culture of *Nitrospira inopinata* has been deposited in the JCM (accession no. JCM 31988). *Nitrosomonas europaea* ATCC 19718 was cultivated in the same basal AFW medium at pH 8.5 and supplemented with 2.5 mM total NH₄Cl. *Nitrospira moscoviensis* was cultivated at 37 °C in mineral nitrite medium (NOM)⁶⁶ amended with 5 mM NO₂[−]. Basal NOM contained (per litre of deionized water): 10 mg CaCO₃, 0.5 g NaCl, 50 mg of MgSO₄ × 7H₂O, 0.15 g KH₂PO₄, 10 mg NH₄Cl, 0.034 mg MnSO₄ × H₂O, 0.05 mg H₃BO₃, 0.07 mg ZnCl₂, 0.0726 mg Na₂MoO₄ × 2H₂O, 0.02 mg CuCl₂ × 2H₂O, 0.024 mg NiCl₂ × 6H₂O, 0.08 mg CoCl₂ × 6H₂O, and 1 mg FeSO₄ × 7H₂O⁶⁶.

Instantaneous NO and N₂O measurement. *N. inopinata*, *N. moscoviensis*, and *N. europaea* cultures were monitored daily and harvested immediately once all the substrate was consumed (normally ~7–9 days) by centrifugation using 10 kDa-cutoff, Ultra-15 Centrifuge Filter units (Amicon, Darmstadt, Germany). About 500 mL of mid-exponential phase culture were harvested per replicate experiment for *N. inopinata*, while 300 L of culture was used per replicate experiment with *N. moscoviensis* and *N. europaea*. Harvested biomass was washed twice with substrate-free medium and then resuspended in 10 mL of the same substrate-free medium. The biomass was then transferred into a 10 mL, double-port MR chamber (allowing no headspace) that was fitted with two MR injection lids and two glass coated stir bars. All MR experiments were performed in a recirculating water-bath at 37 °C. O₂ uptake was

measured using a OX-MR oxygen microsensor (Unisense, Aarhus, Denmark). N₂O and NO concentrations were measured using an N₂O-MR sensor (Unisense) and an ami700-NO sensor (Innovative Instruments, Inc., Tampa, USA), respectively. Substrate (NH₄⁺ or NO₂[−]) were injected into the chamber via an injection port using either a 10-μL or 50-μL syringe (Hamilton, Reno, USA) fitted with a 26G needle. A 250-μL syringe (Hamilton, Reno, USA) fitted with a 26 G needle was also used to withdraw small aliquots (150 μL) from MR chambers during experiments to measure NH₄⁺ and NO₂[−] concentrations, and sterile media was always backfilled. Starting metabolite concentrations were inferred from injected amounts; otherwise, all NH₄⁺ and NO₂[−] concentrations were measured. NH₄⁺, NO₂[−], and NO₃[−] concentrations were quantified photometrically with the Berthelot reagent, acidic Griess reagent, and VCl₂/Griess reagent, respectively^{24,33}, using an Infinite 200 Pro spectrophotometer (Tecan Group AG, Maennedorf, Switzerland). NH₄⁺ injections into the 10 mL MR chamber were always 250 μM for *N. inopinata* and 2 mM for *N. europaea*. NO₂[−] injections were 1 mM for *N. moscoviensis* and 2.5 mM for *N. inopinata*. Higher concentrations of NO₂[−] were used for *N. inopinata* due to the comparatively low apparent affinity of *N. inopinata* for NO₂[−] ($K_{m(\text{app})} = 449.2 \pm 65.8 \mu\text{M}$) compared to its very high affinity for ammonia ($K_{m(\text{app})} = 63 \pm 10 \text{ nM NH}_3$)³³. Further, it was not feasible to measure NO₂[−] oxidation from fully oxic conditions to anoxic conditions due to the slow rate of NO₂[−]-dependent O₂ uptake and the poor affinity of *N. inopinata* for NO₂[−]. We expected no cell doubling during the MR experiments, as the doubling time of all strains (10–40 h) is significantly greater than the duration of the longest MR trace (~2 h). The OX-MR and N₂O-MR sensors were plugged directly into a microsensor multimeter while the ami700-NO sensor was polarized using a One-Channel Free Radical Analyzer (World Precision Instruments, Sarasota, USA). All electrodes were polarized for >1 day prior to use and calibrated according to the manufacturer's instructions. All data were logged on a laptop via the microsensor multimeter using SensorTrace Logger software (Unisense). The output from One-Channel Free Radical Analyzer was run into the microsensor multimeter using a BNC/leno adapter. Abiotic controls were performed in triplicate as described above but with heat-killed cells (autoclaved at 121 °C for 20 min) resuspended in sterile anoxic medium. Anoxically prepared aliquots of NH₄⁺, NO₂[−] and NH₂OH were injected into the MR chamber through the injection port using a 10 μL syringe (Hamilton, Reno, USA). Anoxic AFW, NH₄⁺, NO₂[−], and NH₂OH were prepared by sparging the solutions with N₂ gas for 1 h prior to use.

PTIO inhibition. Batch and micro-respirometry inhibition experiments with the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; purity >98%; TCI, Germany) were performed using exponential-phase cultures of *N. inopinata* and *N. moscoviensis* grown in AOM medium or mineral nitrite medium (NOM)⁶⁶, respectively. For the batch experiments, cells were harvested by centrifugation (8000 × g, 20 min, 20 °C), washed once (10 mg L^{−1} CaCO₃ AOM medium for *N. inopinata* and NO₂[−]-free NOM medium for *N. moscoviensis*) and resuspended undiluted in the same medium containing either 1 mM NH₄Cl (*N. inopinata*) or 1 mM NO₂[−] (*N. moscoviensis*). The cultures was aliquoted (20 mL) in glass serum bottles (60 mL) sealed with crimp caps and quadruplicates were incubated in the dark at 37 °C in the presence of 0, 3.3, 10, 33, 100, and 330 μM PTIO, respectively. Only the concentrations of PTIO that inhibited *N. inopinata* were tested on *N. moscoviensis* (in addition to the unamended control) - 0, 33, 100, and 330 μM PTIO. PTIO was added in the respective amounts as a 10-mM stock solution in autoclaved MilliQ water. Abiotic controls with 0 and 330 μM PTIO and controls with heat-killed cells with 0 μM PTIO were run in triplicate. NH₄⁺, NO₂[−], and NO₃[−] concentrations were quantified with the Berthelot reagent, acidic Griess reagent, and VCl₂/Griess reagent, respectively^{24,33}, using an Infinite 200 Pro spectrophotometer (Tecan Group AG, Maennedorf, Switzerland). Activity percentage was calculated by comparing the difference in rate of NH₃ (for *N. inopinata*) or NO₂[−] (for *N. moscoviensis*) oxidation during the linear section of the substrate oxidation curve between the non-inhibited control culture and the cultures exposed to the various concentrations of PTIO.

For the micro-respirometry-based PTIO inhibition experiments, 360 mL of exponential phase culture was harvested using 10 kDa-cutoff, Ultra-15 Centrifuge Filter units as described above, washed twice with N-free medium, homogenized by vigorous vortexing, and split into 25 2.5 mL aliquots. Individual aliquots were then amended with 0, 33, 100, 330, or 1000 μM PTIO and incubated at 37 °C for at least 2 h. For each experiment, an aliquot was transferred into a 2 mL, single-port MR chamber containing a glass-coated stir bar. O₂ uptake was measured using OX-MR oxygen microsensors in a recirculating water-bath at 37 °C and stirring at 400 RPM. NO₂[−] was injected into the chamber as described above. Viability of the harvested biomass over the length of the entire experiment (~8 h) was confirmed by measuring the rate of substrate-dependent O₂ uptake in biomass incubated at 37 °C without PTIO at the end of the experiment. To determine if removal of PTIO after PTIO treatment restored activity (as being expected if inhibition occurred due to NO scavenging), we harvested the biomass treated with inhibitory concentrations of PTIO using Ultra-15 Centrifuge Filter units, washed it twice with NO₂[−]-free mineral medium using the same Ultra-15 Centrifuge filter units, resuspended it in NO₂[−]-free mineral medium, and transferred it to a 2 mL MR chamber for re-measurement of substrate-dependent O₂ uptake. To control for activity loss due to biomass loss or additional centrifugation, biomass from the 0 μM PTIO treatment group was treated identically. All experiments were run in triplicate. Activity percentage was calculated by comparing the difference in rate of

NH₃ (for *N. inopinata*) or NO₂[−] (for *N. moscoviensis*) oxidation during the linear section of the substrate oxidation curve between the non-inhibited control culture and the cultures exposed to the various concentrations of PTIO.

N₂O yield. Late exponential-phase cultures (containing ~0 μM NH₄⁺ and ~350 μM remaining NO₂[−]) of *N. inopinata* grown in FWM (described above) were transferred at 10% (final volume of 139 mL) to new FWM containing ~1.1 mM NH₄⁺ and aliquoted into glass serum bottles (~255 mL) sealed with butyl rubber stoppers and crimp caps. Quadruplicates were incubated in the dark at 37 °C under a fully oxic atmosphere, with lab air as the headspace, and at low O₂ conditions (~0.9% O₂ with N₂ as the balance, equivalent to 1.8 μM dissolved O₂), respectively. A starting O₂ headspace concentration of ~0.9% in the hypoxic vials was chosen so the starting mol ratio of NH₄⁺ to O₂ was 2.2:1, resulting in O₂-limiting growth conditions. Hypoxic conditions were achieved by sparging the sealed serum bottles with O₂-free N₂ gas for 1 h and then backfilling the headspace with 4.5 mL of lab air. The final concentration of O₂ in the headspace of the hypoxic vials at the beginning of the experiments was 0.89% as verified with a needle-piercing OX-NP O₂ microsensor (Unisense). In addition, abiotic controls containing 1.1 mM NH₄⁺, 250 μM NO₂[−], and 1 μM NH₂OH with and without heat-killed *N. inopinata* cells were run in parallel to control for abiotic formation of N₂O. Gas samples (15 mL) of the headspace were taken from the sealed serum bottles using a sterile syringe and transferred into sealed 12 mL exetainers for N₂O and O₂ analysis. The serum bottles were backfilled with 15 mL of O₂-free N₂ gas after each sampling. N₂O was quantified using a TRACE GC Ultra series gas chromatograph (ThermoFisher Scientific, Waltham, USA) equipped with a pulse discharge detector (ThermoFisher Scientific, Waltham, USA), a Porapak N column, and a AI/AS1310 autosampler (S + H Analytik GmbH, Moenchengladbach, Germany). Final calculated N₂O concentrations take into account N₂O loss due to sampling and are N₂O emissions above experimentally determined background (atmospheric) N₂O levels. NH₄⁺, NO₂[−], and NO₃[−] concentrations were quantified as described previously.

For *N. moscoviensis*, late-exponential phase cultures were transferred at 10% to new NOM containing ~1.0 mM NaNO₂ and aliquoted into glass serum bottles sealed with butyl rubber stoppers and crimp caps. Triplicates were incubated in the dark at 37 °C under a fully oxic atmosphere, with lab air as the headspace, and at hypoxic conditions (~1.0% O₂). Gas samples of the headspace were taken at the beginning of the experiment and once all of the substrate was depleted, so no backfilling was necessary after sampling. N₂O was quantified as described above. Final calculated N₂O concentrations are N₂O emissions above experimentally determined background (atmospheric) N₂O levels.

Analysis of N₂O isotopic signatures. For analysis of the isotopic composition of the N₂O in the headspace of the *N. inopinata* vials, 4 mL of the headspace gas was transferred to 120 mL glass flasks filled with helium at 50 kPa overpressure. Before filling with helium, the flasks had been evacuated and flushed with helium four times. N₂O was analyzed with an isotope ratio mass spectrometer (IRMS, IsoPrime 100, Elementar Analysensysteme, Hanau, Germany) coupled to an online pre-concentration unit (TraceGas, Elementar Analysensysteme) as described in detail in ref. ⁶⁷. Briefly, for pre-concentration, the N₂O was transferred to a cold trap in liquid nitrogen with a transfer time of 20 min. Water, CO₂, and any potential CO in the sample gas were removed with magnesium perchlorate, carboxorb, and a CO oxidation catalyst (Sofnocat), respectively. Then the sample gas N₂O was cryo-focused on a second cold trap, before the sample was remobilized again and separated isothermally at room temperature on a capillary column (PoraPLOT Q, 30 m length × 0.32 mm inner diameter, 10 μm coating, Varian). After GC separation, the N₂O was introduced in a stream of helium to the IRMS via an open-split in continuous-flow mode. Mass-to-charge ratios (*m/z*) of N₂O at 44 (¹⁴N¹⁴N¹⁶O), 45 (¹⁴N¹⁵N¹⁶O), ¹⁵N¹⁴N¹⁶O, and ¹⁴N¹⁵N¹⁷O), and 46 (¹⁴N¹⁴N¹⁸O), as well as NO⁺ fragment ions of N₂O at *m/z* 30 (¹⁴N¹⁶O⁺) and 31 (¹⁵N¹⁶O⁺) were measured simultaneously by the IRMS. The values of δ¹⁵N_{bulk}, δ¹⁵N_α, and δ¹⁸O were calculated from *m/z* 44, 45, and 46, including a ¹⁷O correction according to ⁶⁸. The δ¹⁵N_β value was calculated as δ¹⁵N_β = 2·δ¹⁵N_{bulk} − δ¹⁵N_α, and ¹⁵N site preference (SP) as SP = δ¹⁵N_α − δ¹⁵N_β. Calibration was performed as described in ref. ⁶⁷. Reproducibility was 0.2‰ for δ¹⁵N_{bulk}, 0.3‰ for δ¹⁸O, and 0.4‰ for δ¹⁵N_α and δ¹⁵N_β.

Comparative genomics. Genes encoding NirK, the NorCBQD complex, NorSY complex, CytL, CytS, and NcyA were initially identified by blastp and subsequently examined phylogenetically for annotation. Protein-coding genes from genomes of interest were screened using blastp against databases constructed from previously published lists⁶. For each gene set of interest, blastp results (default parameters) were filtered for queries that hit database entries with at least 70% of the query length. These putative genes were then aligned to the sequences that were used to construct the blastp databases using mafft⁶⁹ and examined for phylogenetic placement using FastTree⁷⁰. Neighborhoods of *nor* genes (*norCBQD* and *norSY*) were manually examined for synteny and scrutinized for genes missed by the blastp search. Alignments of CytL and CytS were further examined for the presence or absence of the diagnostic heme cross-linked lysine⁴⁹ to distinguish phylogenetic clades of CytL from CytS. Putative *nirK* genes were aligned to a set of previously published multicopper oxidase genes⁵³ and manually examined for phylogenetic placement into recognized clades of *nirK*.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The proteomics data used for Supplementary Fig. 3 is publicly available at the PRIDE/ProteomeXchange database under the accession code PXD013103 (ref. ²⁴). The source data underlying Figs. 3–6 and Supplementary Figs. 1, 2, 4–9 are provided as a Source Data file.

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Author contributions

H.D. and M.W. conceived the study. H.D., M.W., and K.D.K. wrote the manuscript with input from all authors. K.D.K., M.Y.J., and C.J.S. performed the micro-respirometry and batch incubations. J.V. and P.P. performed the PTIO batch experiments. S.L. performed the batch *N. moscoviensis* experiments. C.H. made the comparative genome analysis. L.Y. S. assisted with data analysis. A.R., H.W., and N.B. performed the GC and N₂O site preference measurements.

Additional information

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