Title: Oxygen production by an ammonia-oxidizing archaeon

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14 **Abstract:**

15 Ammonia-oxidizing archaea (AOA) are one of the most abundant groups of microbes in the world's
 16 oceans and are key players in the nitrogen cycle. Their energy metabolism, the oxidation of ammonia
 17 to nitrite, requires oxygen. Nevertheless, AOA are abundant in environments where oxygen is
 18 undetectable. In incubation experiments, where oxygen concentrations were resolved to the
 19 nanomolar range, we show that *Nitrosopumilus maritimus* produces oxygen (O₂) and dinitrogen (N₂).
 20 The pathway is not completely resolved, but it has nitric oxide as a key intermediate. Part of the
 21 oxygen produced is directly used for ammonia oxidation, while some accumulates in the surrounding
 22 environment. *N. maritimus* joins a small handful of organisms known to produce oxygen in the dark,
 23 and based on this ability, we re-evaluate their role in oxygen-depleted marine environments.

25 **Main Text:**

26 Ammonia-oxidizing archaea (AOA) are only known to oxidize ammonia to nitrite using oxygen: NH₃
 27 + 1.5 O₂ → NO₂⁻ + H₂O + H⁺ (1, 2). Yet, AOA are highly abundant in environments with very low
 28 or even undetectable oxygen concentrations such as marine oxygen-minimum zones (OMZs) (3–5).
 29 The role of AOA in such environments is enigmatic as they have no known anaerobic metabolism.
 30 We used trace luminescence oxygen sensors (hereafter: optodes) (6) to explore the physiology of
 31 AOA at low nanomolar oxygen concentrations and functional anoxia (oxygen levels below detection)
 32 as typically found in OMZs (7, 8), discovering oxygen production by the marine AOA *Nitrosopumilus*
 33 *maritimus* SCM 1(9). Dark, non-photosynthetic, oxygen production is rare in nature with three known
 34 pathways including chlorite dismutation during perchlorate/chlorate respiration (ClO₂⁻ → Cl⁻ + O₂),
 35 detoxification of reactive oxygen species (e.g. H₂O₂ dismutation) and nitric oxide dismutation (2NO₂⁻
 36 → 2NO → N₂ + O₂) (10). While not fully resolved, we show that the pathway of oxygen production

by *N. maritimus* is none of these and thus novel. Given the abundance of *N. maritimus* in oxygen-sparse environments, anaerobic oxygen production may be common in nature. We also show that oxygen production is linked to N₂ production and thereby identify a previously unknown and potentially environmentally significant N₂ production pathway.

We first grew axenic cultures of *N. maritimus* aerobically as an ammonia oxidizer. The cultures were then sparged with argon to oxygen levels below 5μM, where the remaining oxygen was consumed by *N. maritimus* through continued ammonia oxidation, indicating physiologically active cells. Surprisingly, after oxygen was completely consumed, it immediately started to increase again (Fig. 1A). A series of additions of oxygen-saturated water showed the same recurring pattern: oxygen was consumed and increased directly thereafter (Fig. 1A). When no oxygen additions were made, oxygen build-up continued over hours and reached levels of 100-200nM (Fig. 1A). This pattern was observed reproducibly in multiple incubations carried out over 2 years.

In comparison, no oxygen build-up was detected in filtered abiotic controls or when cells were killed by the addition of mercuric chloride (Fig. S1) ruling out the possibility of abiotic oxygen production or intrusion of oxygen into the incubation bottle. Contamination by oxygen intrusion was further ruled out with incubations in an anaerobic chamber showing the same trend of oxygen production (Fig. S2). Involvement of medium components (e.g. HEPES, EDTA) in oxygen production was also excluded (Fig. S3), and furthermore, oxygen microelectrodes, which make use of a different oxygen measurement principle, showed the same patterns of oxygen increase as the optode measurements (Fig. S4). Incubations with *N. maritimus* in medium containing pyruvate showed no difference compared to incubations without pyruvate (Fig. S5). This experiment rules out oxygen production by H₂O₂ dismutation ($\text{H}_2\text{O}_2 \rightarrow \text{H}_2 + \text{O}_2$) as pyruvate reacts with and removes H₂O₂ via an abiotic decarboxylation reaction (11).

As described in more detail below, we measured nitric oxide (NO) concentrations with a microelectrode and found that NO slightly interferes with the optode measurements of O₂ (Fig. S6) but not the O₂ microelectrode measurements. Therefore, when optode O₂ concentrations and NO were simultaneously measured, a NO correction was applied to the O₂ measurements. The correction, however, was relatively small (0-17%) and fully predictable from the NO concentrations. This correction was only applied when NO and O₂ were simultaneously measured, recognizing that other optode O₂ measurements may be slight overestimates (depending on the NO concentration) of the actual O₂ concentration (Fig. S7).

Despite this small interference on our oxygen measurements, *N. maritimus* clearly produces oxygen when the culture reaches anoxia. We hypothesize that oxygen accumulates as a net balance of simultaneous oxygen production and oxygen consumption by ammonia oxidation, where plateauing oxygen concentrations over time represent a balance between these processes. To test this hypothesis, cyanide (0.5 mM) was added to the oxygen-producing culture. Cyanide inhibits oxygen respiration by the heme-copper oxygen reductase, and thus inhibits ammonia oxidation (12, *supplementary information*). Upon cyanide addition, and after an initial lag phase, oxygen concentrations steadily increased at rates ca. 5 times higher (65 ± 12 nmol/L/h) than before cyanide addition (14 ± 2 nmol/L/h) (Fig. 1B). In similar experiments where NO was also measured, O₂ increase was uncoupled from NO concentration after cyanide addition (Fig. S8). Overall, these results are consistent with the hypothesis that, in the absence of cyanide, some portion of oxygen production by *N. maritimus* is utilized within the cells and does not accumulate into the surroundings.

We tracked the conversion of ¹⁵N-labelled ammonium to nitrite to directly explore if *N. maritimus* continues to oxidize ammonia while producing oxygen. In this experiment, cell cultures were washed to reduce the high nitrite background that accumulated (ca. 1 mM) during normal aerobic growth. After this, ¹⁵N-ammonium (I: 5 μM and II: 25 μM) was added as well as a small

amount of ^{14}N -nitrite to “capture” any produced ^{15}N -nitrite from further transformations. The labelling experiments showed continued ammonia oxidation to nitrite during oxygen production (Fig. 2). These results, consistent with the cyanide addition experiments, confirmed that ammonia oxidation occurs together with oxygen production. Furthermore, the rates of ammonia oxidation in these duplicate experiments were 46 (I) and 39 nM/h (II) (table 1), requiring an oxygen production rate of 69 and 60 nM/h respectively, given the stoichiometry of ammonia oxidation ($\text{NH}_3 + 1.5\text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+$). Oxygen accumulated at an average rate of only 1.2 nM/h in both experiments. Our results further demonstrate that most of the oxygen produced by *N. maritimus* was immediately consumed through ammonia oxidation. The cell density in these incubations was 1.3×10^7 cells mL^{-1} , and therefore, the average ammonia oxidation rate per cell was 3-3.5 amol/cell/h. In marine OMZs with typical AOA cell densities of about $1\text{-}10 \times 10^4$ cells mL^{-1} this would correspond to ammonia oxidation rates of 1-10 nM/d, which are in the range of anammox rates in open-ocean oxygen-minimum zones, the so far only known anaerobic ammonia oxidation process that is considered to be relevant in marine OMZs (8).

To explore for translational changes associated with oxygen production, we performed differential proteomic analysis comparing *N. maritimus* performing standard aerobic metabolism to its metabolism during oxygen production. The proteome translation profile during aerobic metabolism matched earlier findings (14, see supplementary information for details). However, compared to the mid-log phase during normal aerobic growth, 88 of the 1453 recovered proteins had significantly increased abundances when the culture produced oxygen, while 11 proteins were significantly decreased in abundance ($P < 0.05$; table S1).

The plastocyanin Nmar_1665, the multicopper oxidase type 3 Nmar_1354 and the putative nitroreductase Nmar_1357, were among the most up-regulated proteins during oxygen production, suggesting that they could play a role in the oxygen-production pathway (Fig. S9). Indeed, nitric

oxide processing has been suggested for the gene cluster Nmar_1352-1357 (13, 14), which would be consistent with the tight coupling of oxygen production and nitric oxide accumulation as explored below. The 17 small blue Cu-containing plastocyanins encoded by *N. maritimus* have a proposed function in hydroxylamine (NH₂OH) oxidation (14). Of these, Nmar_1665 was the only plastocyanin with a significant upregulation under oxygen production, while all other plastocyanins were abundant under both metabolic modes. Other proteins that are either significantly up or down regulated include different transcriptional regulators: e.g members of the AsnC family (Nmar_1524 [up], Nmar_1292 [up], Nmar_1628 [down] (Fig. S9)). Archaeal AsnC regulatory proteins have a function in the regulation of central and energy metabolism, and a role in the switch between aerobic and anaerobic metabolism has been suggested (15, 16). The NADH dehydrogenase 30 kDa subunit Nmar_0278 was significantly down regulated. Otherwise, no proteins with functions in energy or carbon metabolism changed significantly in abundance.

Overall, the few significant changes in the proteome between aerobic respiration and oxygen production suggest that most proteins are needed and active under both metabolic modes. However, some of the upregulated proteins could be involved in catalyzing and regulating oxygen production in AOA.

We now explore possible metabolic pathways for dark oxygen production in *N. maritimus*. Of the three known pathways of dark oxygen production, we rule out perchlorate/chlorate respiration as our cultures were perchlorate/chlorate/chlorite free. Furthermore, as discussed above, we also rule out hydrogen peroxide dismutation as a source of oxygen. As *N. maritimus* metabolizes nitrogen and accumulates NO under normal aerobic ammonia oxidation (17), NO dismutation becomes a potential source of oxygen in our experiments. So far, NO dismutation is only known among the NC10 bacteria (10). These organisms are methane oxidizers and generate NO for dismutation to oxygen and dinitrogen (18), where the oxygen is used to oxidize methane. Because oxygen production and

consumption are tightly coupled, methane-oxidizing NC10 bacteria are not known to liberate free oxygen into the environment (18).

Using NO microelectrodes, we noted that NO and oxygen production were mostly tightly coupled (Fig. S7) (although the coupling was less tight in other cases; Fig. S8)). Furthermore, when the NO scavenger PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) was added, oxygen production ceased (Fig. S10). Taken together, these results suggest that NO is a crucial intermediate in oxygen production.

We used ^{15}N -labelled nitrite to further unravel the pathways of nitrogen and oxygen cycling during oxygen production by *N. maritimus*. In incubations with added ^{15}N -nitrite, $^{30}\text{N}_2$ was produced during oxygen production (Fig 3A, S12 and 13), while no formation of $^{29}\text{N}_2$ was detected (Fig. 3B). Dinitrogen production by *N. maritimus* or other AOA isolates has not previously been reported. Our results furthermore show that both nitrogen atoms in the N_2 originated from nitrite, with none coming from ammonium. This result was confirmed by incubations with ^{15}N -ammonium, where no immediate conversion of ^{15}N -ammonium to $^{29}\text{N}_2$ or $^{30}\text{N}_2$ was detected (Fig. 3A, B). Instead, ^{15}N -ammonium was most likely converted to nitrite and diluted into the large existing nitrite pool in this experiment. In contrast, when ^{15}N -labelled ammonium was added to washed cultures with a small nitrite pool (5 and 25 μM), the ^{15}N -nitrite produced from ammonia oxidation was further converted to N_2 (Fig. 2B and S11) consistent with our experiments with ^{15}N -labelled nitrite.

Thus far we have shown that NO is a likely intermediate in oxygen production and that N_2 is produced together with O_2 by *N. maritimus*. Rates of O_2 accumulation and N_2 production from the different incubations shown in Figs. 2 and 3 are summarized in table 1. These results would generally be consistent with NO dismutation as a source of both N_2 and O_2 , where NO is produced from the reduction of nitrite. In incubations with added ^{15}N -nitrite, however, oxygen accumulation exceeded N_2 production in the first 20 h (incubations 3 and 4 in Table 1, Fig. 3c, S12 and S13), demonstrating

a decoupling of O₂ and N₂ production in this phase of the experiment. As net rates of O₂ accumulation may underestimate gross rates of O₂ production, as explored above, and as N₂ production in our experiments is a gross production rate, there is an imbalance between the production rates of O₂ and N₂. Such an imbalance would be inconsistent with O₂ and N₂ production directly from NO dismutation. In this case the O₂ accumulation rates should not exceed N₂ production rates, but the imbalance we measure suggests that further intermediate(s) must exist between NO, and N₂ and O₂ production. We suggest that N₂O may be such an intermediate, where $2\text{NO} \rightarrow \text{N}_2\text{O} + 0.5\text{O}_2$.

Indeed, in our incubations supplied with ¹⁵N-nitrite, ⁴⁶N₂O accumulated before ³⁰N₂ production accelerated (Fig. 3D), and the rates of N₂ production and N₂O accumulation taken together in the first 20 h match the O₂ accumulation rates within the uncertainties (table 1). Furthermore, the dismutation of NO(aq) to O₂(aq) and N₂O(aq) is thermodynamically favorable ($\Delta G^{0'} = -165 \text{ KJ/mol O}_2$). However, for this pathway to occur, an unknown N₂O reductase would need to be present in the genome of *N. maritimus* (14). N-nitrosating hybrid formation, in which one N atom from NO₂⁻ and one from NH₄⁺ (or an intermediate of ammonia oxidation) combine to form N₂O, has been proposed as a possible source for N₂O production in AOA (19), but the isotopic signature of the N₂O produced in our experiments (⁴⁶N₂O) does not support this pathway (expected ⁴⁵N₂O). In incubations supplied with ¹⁵N-ammonium and a small nitrite pool (Fig. 2), N₂O accumulated transiently as well (Fig. S14). In these incubations N₂ production far exceeded O₂ accumulation and no N₂O accumulation would be required for a mass balance. This is not surprising as high rates of ammonia oxidation (Table 1, Fig 2) indicate that O₂ accumulation rates in these experiments far underestimate gross rates of O₂ production as explored above. This does not mean that N₂O was not an intermediate in these experiments, only that these incubations did not demonstrate an initial imbalance between O₂ and N₂ production.

179 To summarize, like for NO dismutation in NC10 bacteria, O₂ production in *N. maritimus* has
 180 NO as an intermediate and produces N₂. However, unlike for NC10 bacteria, our results suggest that
 181 the pathway of O₂ production employed by *N. maritimus* has an extra intermediate that may be N₂O.
 182 A proposal for the metabolic pathway associated with oxygen production in *N. maritimus* is shown
 183 in figure 4. While ammonia oxidation to nitrite is accomplished by the O₂ produced by *N. maritimus*,
 184 the conversion of nitrite to N₂ requires reducing equivalents regardless of the O₂ production pathway.
 185 The required electrons can partly, but not fully, be obtained from the ongoing oxidation of ammonia.
 186 Another source of electrons could be intra- or extracellular organic matter produced during normal
 187 aerobic ammonia oxidation (20) or, *in situ*, by dissolved organics available in the water column.

188 By showing that the O₂ production pathway in AOA is coupled to N₂ production, we also
 189 uncovered a new, potentially environmentally significant, pathway of N₂ production. Furthermore,
 190 by converting ammonium through nitrite to N₂, AOA perform a so far unrecognized pathway of
 191 anaerobic ammonia oxidation. ¹⁵N-tracer experiments currently performed to measure N-cycling
 192 rates in marine oxygen-depleted environments would overlook this pathway and account for its N₂
 193 production as canonical denitrification and/or anammox.

194 AOA are one of the most abundant groups of microbes in the global ocean and key players in
 195 the marine nitrogen cycle, also in low-oxygen environments. Thus, a widely distributed oxygen-
 196 producing pathway by AOA could have far-reaching implications for the microbial ecology and
 197 biogeochemical cycling in oxygen-depleted environments, including the possibility that some of the
 198 produced could be used by other microbial cells. The discovery of an anaerobic oxygen-producing
 199 pathway in AOA can explain the presence and role of AOA in such environments solving a
 200 longstanding enigma.

201

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Acknowledgments:

We thank Annie Glud for assistance with microelectrode measurements and providing microelectrodes. **Funding:** This work was supported by the Villum Foundation, Denmark (Villum Young Investigator Grant No. 25491 to BK and Villum Investigator Grant No. 16518 to DEK) and the Independent Research Fund Denmark (Grant No. 14181-00025 to DEC). **Author contributions:** B.K. and D.E.C. designed the experiments. B.K. performed the experiments and analyzed data with input from M.L, L.B., M.K., B.T. and D.E.C.. N.J. performed proteomics. B.K. and D.E.C. wrote the manuscript, with contributions and approval from all other authors. **Competing interests:** The authors declare no conflict of interest. **Data and materials availability:** All data is available in the main text or the supplementary materials.

Supplementary Materials:

Materials and Methods

Figures S1-S14

Tables S1-S2

References (21-30)

Fig. 1. Oxygen production by *N. maritimus*. a) after supplied oxygen is consumed, oxygen concentrations immediately start to increase again (0-8h). The oxygen concentration increases over time when no oxygenated water is added (8h-20h). b) cyanide additions lead to a strong increase in oxygen production. Two parallel incubations showed the same pattern as observed in 1a: oxygen increased immediately after added oxygen had been consumed and accumulated over time when no oxygen additions were performed. After the additions of cyanide (0.5mM final concentration, shaded areas), oxygen accumulations strongly increased. Stars: additions of oxygen saturated water, arrows: addition of cyanide. Colors: black and grey lines represent parallel incubations.

322

323 Fig. 2. Ammonia oxidation to nitrite and N_2 during oxygen production by *N. maritimus*. A) ^{15}N - nitrite
324 production from ^{15}N -ammonium for two sets of incubations of washed *N. maritimus* culture.
325 Incubation I contained a ^{14}N -nitrite pool of 5 μM and incubation II had a ^{14}N -nitrite pool of 25 μM .
326 ^{15}N -nitrite production continued after supplied oxygen was consumed (10h). B) Total N_2 production
327 in incubations I and II. Results include $^{28}\text{N}_2$ from ^{14}N -nitrite as well as $^{30}\text{N}_2$ and $^{29}\text{N}_2$ from added ^{15}N -
328 ammonium that was converted to ^{15}N -nitrite and partly captured in the small ^{14}N -nitrite pool before
329 further conversion to $^{30}\text{N}_2$ and $^{29}\text{N}_2$ (results in Fig. S11). C) Oxygen accumulation in a subset of
330 exetainers. Exetainers A and B belong to incubation I, and C, D and E to incubation II. Error bars
331 represent the standard deviation of 3 replicates.

Fig. 3. N_2 production and N_2O accumulation by *N. maritimus* during simultaneous oxygen production. After sparging with argon, *N. maritimus* culture was incubated with either ^{15}N -nitrite or ^{15}N -ammonium and the production of (A) $^{30}\text{N}_2$ and (B) $^{29}\text{N}_2$ were tracked in two independent sets of incubations for each tracer addition (3-4 replicates each). N_2 production was only detected in incubations with ^{15}N -nitrite. For the corresponding oxygen measurements see fig. S12 and 13. C) Oxygen accumulation and $^{30}\text{N}_2$ production for a single replicate of the incubation series 1). The insert shows $^{30}\text{N}_2$ production in the first 20h. Disturbances in the oxygen time series at T=0, 3, 10,

340 15.5, 23, 34 and 38h correspond to the time points when samples for N₂ analysis were taken, which
 341 led to slight oxygen intrusion. Gray dots: oxygen, black open squares: ³⁰N₂. D) ⁴⁶N₂O accumulation
 342 in incubations 1) and 2) supplied with ¹⁴N-ammonium and 1 mM ¹⁵N-nitrite. Only ⁴⁶N₂O
 343 accumulated in these incubations with a large ¹⁵N-nitrite pool indicating that all produced N₂O
 344 originated from nitrite. Error bars represent the standard deviation of 4 (incubations 1 and 3) or 3
 345 (incubations 2 and 4) replicates.

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350 Fig. 4. Proposed pathway of oxygen and dinitrogen production in *N. maritimus*. Ammonia oxidation
 351 pathway (gray): for the aerobic oxidation of ammonia to nitrite, oxygen is needed to activate
 352 ammonia oxidation by the ammonia monooxygenase (AMO), additionally four electrons per
 353 oxidized NH_4^+ are transferred to the terminal oxygen reductase, which reduces oxygen. Proposed
 354 oxygen production pathway (black): Nitrite is reduced to nitric oxide by the NirK nitrite reductase
 355 (NIR). Nitric oxide is dismutated to oxygen and nitrous oxide. The accumulating oxygen is partly
 356 consumed during ammonia oxidation. Likewise, part of the nitric oxide may be used for driving the
 357 hydroxylamine oxidation step of the ammonia oxidation pathway. Finally, nitrous oxide becomes
 358 reduced to dinitrogen. This pathway requires four electrons per produced N_2 . These may partly be
 359 supplied by the ammonia oxidation reaction, which in return would reduce the oxygen demand by
 360 the ammonia oxidation pathway. Cu-"P460": the hydroxylamine oxidizing enzyme, unknown:
 361 unknown enzyme.

Table 1: Summary of rates extracted from incubations with $^{15}\text{NH}_4^+$ or $^{15}\text{NO}_2^-$ additions presented in Figs. 2 and 3. Oxygen accumulation rates were taken when the accumulation rate was at its maximum at the start of oxygen production. In incubations I and II oxygen supplied to the culture at the beginning of the incubation was consumed after 10h (Fig. 2C). Therefore, only time points after 10h were used to calculate ammonia oxidation rates during oxygen production. The means of the rates from replicate incubations and its standard deviation are presented. n. d.: not determined, -: no N_2O accumulation was detected. N_2 production rates refer to the total N_2 production, which in case of incubations 1 and 2 equal $^{30}\text{N}_2$ production rates.

Incubation	O_2 accumulation (nM/h)	NH_3 oxidation to NO_2^- (nM/h)	N_2 production (first 20h) (nM/h)	N_2 production (20-40h) (nM/h)	N_2O accumulation (first 20h) (nM/h)
$^{15}\text{NH}_4^+$, 5 μM $^{14}\text{NO}_2^-$ (fig. 2, incubation I)	1.2 (± 0.2)	46 (± 12)	49 (± 12)	-	-
$^{15}\text{NH}_4^+$, 25 μM $^{14}\text{NO}_2^-$ (fig. 2, incubation II)	1.2 (± 0.2)	39 (± 9)	51 (± 6)	3 (± 1)	3 (± 1)
$^{14}\text{NH}_4^+$, 1mM $^{15}\text{NO}_2^-$ (fig. 4, incubation 1)	21 (± 8)	n. d.	9 (± 3)	51 (± 13)	5 (± 2)
$^{14}\text{NH}_4^+$, 1mM $^{15}\text{NO}_2^-$ (fig. 4, incubation 2)	24 (± 8)	n. d.	11 (± 5)	37 (± 20)	22 (± 6)