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# Denitrification and aerobic respiration, hybrid electron transport chains and co-evolution☆

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ABSTRACT

This paper explores the bioenergetics and potential co-evolution of denitrification and aerobic respiration. The advantages and disadvantages of combining these two pathways in a single, hybrid respiratory chain are discussed and the experimental evidence for the co-respiration of nitrate and oxygen is critically reviewed. A scenario for the co-evolution of the two pathways is presented. This article is part of a Special Issue entitled: The evolutionary aspects of bioenergetics systems.



## 1. Introduction

After the evolution of oxygenic phototrophy, molecular oxygen has become the major electron acceptor on Earth; it is responsible for the oxidation of most of the organic matter originating from primary production. Bacteria are also able to use several other electron acceptors for respiration. Among these electron acceptors, nitrate is common in the environment and has an oxidative potential which approximates that of oxygen. The most studied and evolutionary most widespread form of nitrate respiration is known as denitrification. In this process, nitrate is reduced stepwise to nitrite, nitric oxide, nitrous oxide and finally nitrogen ($N_2$).

In the past century, mankind has become dependent on the industrial production and agricultural use of chemical fertilizers. This has created an important new source of nitrate for the biosphere. Part of the applied fertilizer is oxidized to nitrate by nitrifying bacteria, detaches from the negatively charged clay particles and is washed into the surface waters via rainwater or irrigation. On top of this, the burning of fossil fuels also contributes fixed nitrogen in the form of ammonia deposition. It is estimated that currently every one out of two nitrogen atoms in the biosphere originates from fertilizers or fossil fuels [1]. This has increased the importance of denitrification relative to aerobic respiration in aquatic habitats. Because denitrification leads to the emission of nitrous oxide (a potent greenhouse gas and ozone scavenger) to the atmosphere, it is currently actively researched by environmental scientists.

Denitrification and aerobic respiration depend on the same core respiratory machinery. This machinery consists of the NADH dehydrogenase (complex I), the quinone pool, the $bc_1$ complex (complex III) and cytochrome *c*. Each of the two pathways adds its own specific modules to this backbone. Aerobic oxidation requires a terminal oxidase (complex IV) which accepts electrons either from cytochrome *c* or the quinone pool. Denitrification consists of four modules: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. Organisms that contain at least two or three of these enzymes and produce nitrous oxide or dinitrogen gas will be referred to as "denitrifiers".

As far as we know, all denitrifiers are also capable of aerobic respiration, and the simultaneous "plugging in" of all modules into the backbone would lead to a highly branched respiratory chain in these organisms. Although the four steps of denitrification operate in series from the perspective of the electron acceptor ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), they operate in parallel from the perspective of the respiratory chain (all accept their electrons from cytochrome *c* or the quinol pool) (see Figs. 1–3).

The discovery of "intra-aerobic" denitrification has complicated matters even further. This process adds yet another module to the already rich inventory, namely the dismutation of nitric oxide into

*Abbreviations:* Complex I, NADH dehydrogenase; Complex III, $bc_1$ complex; Complex IV, terminal oxidase; FMN, flavin-mono-nucleotide; ISP, iron sulfur protein; Mo-*bis*MGD, molybdenum *bis* molybdopterin guanine dinucleotide; Nap, periplasmic nitrate reductase; Nar, membrane bound nitrate reductase; Nir, nitrite reductase; Nod, nitric oxide dismutase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase; Nrf, nitrite:ammonia oxidoreductase; Paz, pseudoazurin; Q, quinol; TAT, twin-arginine translocation

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oxygen and nitrogen ($N_2$) [2] (Fig. 4). The dismutation requires no electrons but would in theory enable the operation of a hybrid denitrification/aerobic respiratory chain. Such as chain starts out with the nitrate and nitrite reduction into nitric oxide. Subsequently, the nitric oxide is dismutated into oxygen and the final part of the chain proceeds aerobically via complex IV. Members of the heme-copper oxidase superfamily or a divergent multicopper oxidase have been implicated in the catalysis of the dismutation. It still needs to be investigated how widespread nitric oxide dismutation is and whether the intracellularly produced oxygen is restricted to serve the oxidation of recalcitrant electron donors such as methane. However, it opens up interesting theoretical possibilities that will partly be explored in this paper.

In the following section the enzymology of the different respiratory modules will be briefly summarized. For more detailed information on individual enzymes, the reader is referred to excellent specialized reviews previously published (see below). Next, the bioenergetics of (combinations of) the different denitrifying and aerobic respiratory chains are discussed in terms of their catabolic energy efficiency. The paper continues with an overview of the current experimental evidence that the two pathways may function simultaneously in one complex respiratory network. Finally, a scenario is presented for a possible shared evolutionary origin of aerobic respiration and denitrification.

## 2. A brief summary of the enzymes, the modules of the denitrifying/aerobic respiratory chain

What follows is a brief introduction of the respiratory backbone and the different modules necessary for aerobic respiration and denitrification. The canonical, best-studied forms of the enzyme complexes are presented (Fig. 2). Deviations are known (e.g. in mononderm bacteria and archaea, both lacking a periplasm [3], Fig. 3) but have only been explored partially. It is likely that even more exceptions will be discovered in the future. To acknowledge the different types of quinols used by different organisms (menaquinol, ubiquinol, etc.), we refer to these molecules as the "quinone/quinol pool."

Both aerobic respiration and denitrification handle compounds that easily give rise to reactive radicals. These can cause oxidative damage to the cell [4]. Many of the enzymes involved also depend on iron for catalysis or electron transfer and even more radicals are formed in the presence of iron (due to the Fenton reaction [5]). Therefore, apart from the core enzymes described here, more enzymatic machinery will be in place to quench the formation of radicals. Because of the highly branched nature of the denitrifying respiratory chain, imbalances in electron donor supply can potentially lead to (temporal) incomplete denitrification and build up of nitrite or nitric oxide. Binding of nitric oxide to specific carrier molecules (such as cytochrome *c*′) and the use of sensors and regulators may help to prevent this scenario [6]. These aspects are not addressed here and we focus on the structural parts of the pathway.

Figs. 1–3 illustrate three examples of the integration of the different modules into the backbone respiratory chain.

### 2.1. NADH dehydrogenase (complex I)

Together with the quinone pool, complex III and cytochrome *c*, NADH dehydrogenase constitutes the backbone of the respiratory chain (reverse electron transport is also possible, but is not discussed further here). Bacterial complex I consists of up to 14 subunits (550 kDa total), encoded by the genes *NuoABCDEFGHIJKLMN* [7,8]. The electrons are transduced from NADH (−0.32 V) to Flavin-mono-

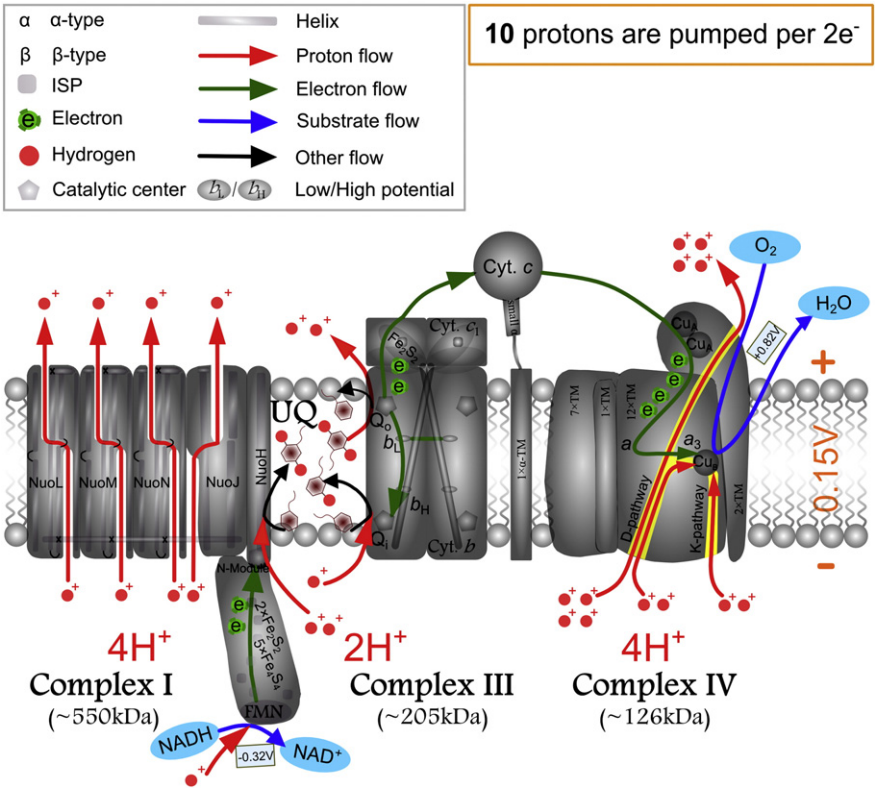


**Fig. 1.** The canonical respiratory chain of oxygen respiration. Electrons are transferred from NADH to complex IV via complex I, ubiquinone/ubiquinol, complex III and cytochrome *c*. Protons are pumped across the membrane by complex I, complex III (Q-cycle) and complex IV. Overall, the contribution to the proton motive force is 10 protons per electron pair.

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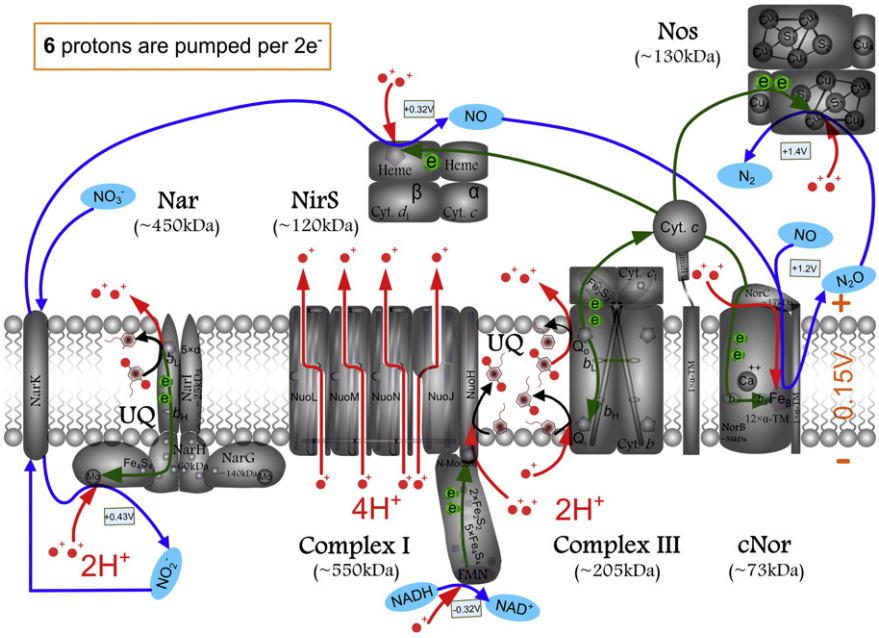


**Fig. 2.** The canonical respiratory chain of denitrification. Electrons are transferred from NADH to NO*x* reductases (e.g. nitrate, nitrite, nitric oxide and nitrous oxide reductases) via complex I, ubiquinone/ubiquinol, complex III and cytochrome *c*. Protons are removed from the cytoplasm by complex I, complex III (Q-cycle) and the cytoplasmic nitrate reductase. Overall, the contribution to the proton motive force is 6 protons per electron pair.

nucleotide (FMN), nine iron sulfur clusters and finally the quinone pool (+0.113 V for ubiquinone) [9]. Conformational change during electron transport leads to the translocation of four protons over the cell membrane per electron pair and the reduction of the quinols leads to the removal of two additional protons from the cytoplasm [10–13].

### 2.2. The $bc_1$ complex (complex III)

The $bc_1$ complex relays the electrons from the quinone pool inside the membrane to cytochrome *c* (+0.23 V) in the periplasm [14]. Bacteria encode many varieties of this complex but it always consists of an integral membrane protein with two or more heme *b* cofactors and at least two periplasmic subunits: the Rieske iron sulfur protein and cytochrome *c* [15–17]. The protons produced during the oxidation of the quinols are set free in the periplasm [18]. Complex III can also run the "Q-cycle" leading to the translocation of an additional proton across the membrane. This is thermodynamically only possible when the membrane potential is not too high (<~0.12 V with ubiquinone). The Rieske iron sulfur protein needs to be translocated to the periplasm in a folded state, requiring the action of the twin arginine translocation (TAT) system [19].

### 2.3. Cytochrome c

In these generally small proteins (normally 10–20 kDa) the heme is covalently bound to cysteine moieties of the protein via sulfur bridges. The covalent bonds are forged in the periplasm and this requires the action of a dedicated cytochrome *c* maturation system [20–25]. As an

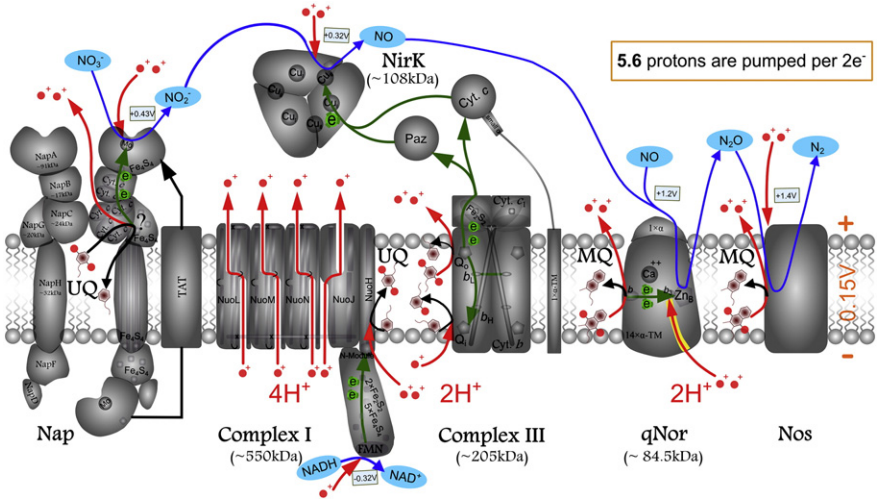


**Fig. 3.** Alternative forms of denitrifying enzyme complexes: the periplasmic nitrate reductase (Nap), copper-type nitrite reductase (NirK) and quinol dependent nitric oxide reductase (qNor). Pseudoazurin (Paz) is an alternative to cytochrome *c* to transport electrons to NirK. Overall, the contribution to the proton motive force is 5.6 protons per electron pair.

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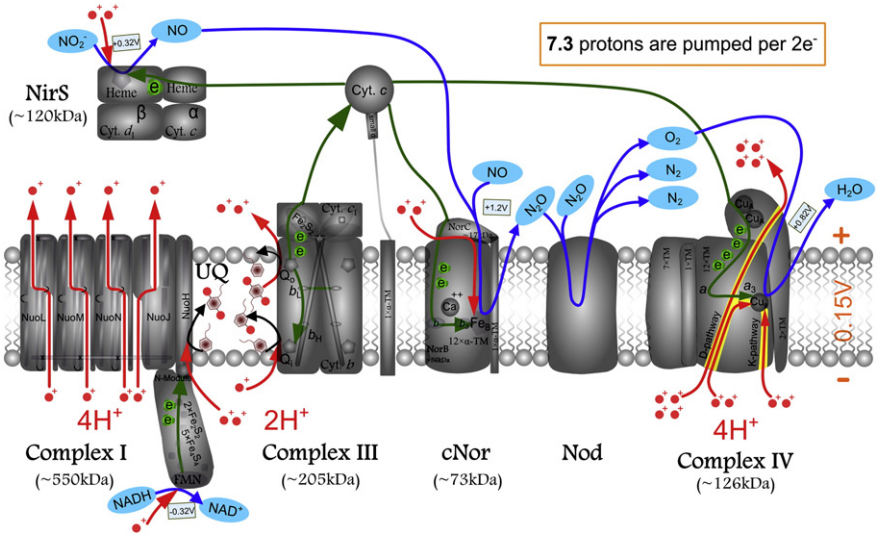


**Fig. 4.** Hybrid respiratory chain combining denitrification via nitric oxide dismutation and oxygen reduction. Electrons are partly transferred from NADH to NirS via complex I, ubiquinone/ubiquinol, complex III and cytochrome *c*. The nitric oxide dismutase (Nod) converts nitric oxide to nitrogen gas and oxygen without receiving any electron. The produced oxygen is then reduced by complex IV, generating proton gradient. Overall, the contribution to the proton motive force is 7.3 protons per electron pair.

alternative to cytochrome *c*, copper based electron carriers such as plastocyanin can be used [26]. Like cytochrome *c*, biosynthesis of these proteins also requires a maturation system.

### 2.4. Terminal oxidase (complex IV)

This complex transduces the electrons from cytochrome *c* (+0.23 V) or quinones (+0.113 V for ubiquinone) to oxygen ($O_2$, +0.8 V). Three non-homologues enzymes perform this step: (a) members of the heme-copper oxidase superfamily, (b) the *bd*-type cytochrome oxidases and (c) the alternative oxidase. The oxygen-reducing terminal oxidases (complex IV) are integral membrane proteins that reduce oxygen at a binuclear active center composed of a high spin heme ($a_3$, $o_3$ or $b_3$) and a copper ion ($Cu_B$). The four protons needed for the reduction of oxygen to water are extracted from the cytoplasm and contribute to the proton motive force [27,28]. The *bd*-type oxidase accepts electrons only from the quinol pool. It is an integral membrane protein with two subunits, two heme *b*'s and one heme *c*. Like the heme-copper enzymes it extracts protons from the cytoplasm [29,30]. The alternative oxidase, finally, is not a membrane protein but reduces oxygen in the cytoplasm. For this reason it contributes to the dissipation of the proton motive force and no energy is conserved. Higher organisms make use of this complex to generate heat. It accepts electrons from the quinone pool and its active site consists of a di-iron center [30]. The varieties of complex IV differ in their affinity for oxygen. High affinity terminal oxidases (e.g. the *bd*-type and the $cbb_3$ heme-copper oxygen reductases) are used by microaerobic organisms.

### 2.5. Nitrate reductase

This enzyme complex catalyzes the first step of denitrification, the two-electron reduction of nitrate to nitrite (+0.43 V). It is a member of the large family of molybdopterin oxidoreductases. The enzyme can either be located in the cytoplasm (NarGH) or in the periplasm (NapAB) [31]. In the first case, more energy is conserved because of the additional consumption of protons from the cytoplasm [32], while in the latter case (NapAB), energy is only conserved by the action of complex I [33,34]. The electrons from quinols are relayed to the molybdopterin (Mo-*bis*MGD) active site by a series of iron–sulfur clusters [35,36]. In some versions of the complex NarGH is located in the periplasm, but is coupled to a complex III-like cytochrome *b* subunit which may still enable the conservation of the energy [37]. The periplasmic form of the enzyme is widespread among bacteria and can fulfill a role in either fermentation, denitrification [33,38] or phototrophy (redox poising the cyclic electron transfer chain) [39]. NarG is much less widespread and used only by denitrifying bacteria (which really depend on the conservation of energy). The translocation of the nitrate reductase to the periplasm requires the action of the TAT system [19].

### 2.6. Nitrite reductase

Two non-homologous enzymes catalyze the one-electron reduction of nitrite into nitric oxide (+0.36 V): the $cd_1$ nitrite reductase encoded by *NirS* (120 kDa for the homodimer) and the copper-type nitrite reductase encoded by *NirK* (108 kDa for the homotrimer). Both enzymes are located in the periplasm and require the action of specific periplasmic chaperones for maturation [40,41]. Cytochrome *c* (+0.23 V) and/or small copper proteins (e.g. pseudoazurin, Paz) can serve as the electron donor for both enzymes [40,42,43]. Because of their periplasmic localization these enzymes do not contribute to the proton motive force directly. It is unknown whether the two apparently isofunctional enzymes confer specific advantages or disadvantages to their host, but they have so far not been found together in the same organism. $Cd_1$ nitrite reductase also reduces oxygen [44,45].

### 2.7. Nitric oxide reductase

These enzymes (~75 kDa) are part of the superfamily of heme-copper oxidase (see complex IV above) [46]. Instead of copper, the active site of nitric oxide reductase contains non-heme iron ($Fe_B$) [47]. Two molecules of nitric oxide are combined, and reduced (+1.18 V) to nitrous oxide with the addition of two electrons. The protons necessary originate from the periplasm so the energy is not conserved [48,49]. Just like the oxygen reductases, different forms of nitric oxide reductase exist: one (encoded by *NorZ*) accepts electrons from quinols and the other (a heterodimer encoded by *NorBC*) from cytochrome *c* [47,50]. All members of the heme-copper oxygen reductases can reduce both nitric oxide and oxygen; only the affinity for either of these substrates differs.

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### 2.8. Nitrous oxide reductase

The final step of denitrification, the reduction of nitrous oxide into nitrogen (+1.36 V), is performed by nitrous oxide reductase, a periplasmic homodimer of 130 kDa (total) encoded by *NosZ* [51]. It accepts electrons from cytochrome *c* and does not conserve the energy. It contains two copper centers ($Cu_A$ and $Cu_Z$) and the electrons are relayed from the former to the latter, where the reduction of nitrous oxide actually takes place. $Cu_Z$ contains four copper atoms bridged by two sulfur atoms and is inactivated by oxygen [52]. Specific chaperones (NosDFZL) are necessary for maturation in the periplasm [53,54].

### 2.9. Nitric oxide dismutase

This enzyme has only recently been postulated to exist, based on the observation that $^{18}O$-labeled $O_2$ was produced from $^{18}O$-labeled nitrite by the bacterium "*Candidatus* Methylomirabilis oxyfera." It was previously implied that members of the heme-copper oxidase superfamily or a divergent multicopper oxidase might catalyze this reaction [2].

## 3. The bioenergetics of denitrification compared to aerobic respiration

Denitrification and aerobic respiration have only slightly different overall redox potentials, so from a theoretical, thermodynamic perspective nitrate and oxygen are almost equally good electron acceptors. However, in reality oxygen is a much better electron acceptor both for bioenergetic and kinetic reasons. From a bioenergetic perspective, much more energy is conserved during aerobic respiration. Per electron pair transduced, four protons are translocated by complex I, two protons are translocated during the reduction and re-oxidation of the quinones (at complex III or IV) and four additional protons are translocated by complex IV. In total, up to 10 protons are translocated. At a membrane potential of 150 mV, approximately 50% of the energy is conserved in the form of a proton motive force, and can be used to generate ATP. In canonical denitrification, at most six protons per pair of electrons are translocated if NAHD is used as electron donor. This difference is caused by the fact that none of the denitrification modules translocates protons. Even the nitric oxide reductase, which can be considered as a form of complex IV does not contribute to the proton motive force. This is caused by the fact that, in spite of the high potential of nitric oxide (1.18 V, higher than that for oxygen reduction) the active site of nitric oxide reductase is a too weak base to "pull" the protons from the cytoplasm [55]. Overall, only 30% of the energy is conserved in the form of a proton motive force (see Fig. 2). Most of the energy is lost in the final part of the denitrification pathway, where the high potential electron acceptors nitric and nitrous oxides do not contribute to the proton motive force. From the perspective of the organism, it does not matter whether it reduces nitrate to nitrite (+0.43 V) or nitrous oxide to nitrogen (+1.36 V), the amount of energy conserved is equal (six protons per electron pair).

When denitrifiers would be able to make use of nitric oxide dismutation, the amount of energy conserved would improve. In that case, 7.3 protons would be translocated per electron pair, leading to 36.5% energy conservation, and from an evolutionary perspective a significant fitness benefits.

Apart from this bioenergetic disadvantage, we would like to hypothesize that denitrifiers also suffer from a kinetic disadvantage. This can be understood as follows: (a) denitrifiers need more different enzyme complexes (in addition to the core four modules) than aerobic organisms (only one additional enzyme); (b) the amount of space in both the membrane and the periplasm is limited. Thus, denitrifiers must have lower numbers of each of the individual complexes per cell, leading to a lower maximum substrate conversion rate and a larger average distance between the donors and the acceptors in each of the different steps of the respiratory chain, which would further decrease the respiration speed.

The number of different modules required for denitrification via nitric oxide dismutation would also amount to four.

These are two important physicochemical constraints that limit the fitness of denitrification compared to aerobic respiration. Since as far as we know all denitrifiers are also capable of aerobic respiration, it is clear that in the presence of oxygen, denitrification appears to make no bioenergetic sense.

In well studied denitrifiers (e.g. *Pseudomonas stutzeri* and *Paracoccus denitrificans*), several regulatory systems are known to shut down denitrification once oxygen is detected. Further, the final step of denitrification, the reduction of nitrous oxide to nitrogen is inhibited by oxygen because oxygen deactivates the responsible enzyme [52].

On the other hand, one may still conceive specific environmental conditions where the co-expression of both pathways in one organism, and even the aerobic activity of denitrification, can be profitable. For example, the oxygen concentration in many natural systems is low, oxygen is not very soluble (<250 μM) and the oxygen consumption rate can be high. This often gives rise to steep oxygen gradients and the occurrence of oxygen limitation. When the production of NADH is more rapid than the supply of oxygen, the co-respiration of nitrate and oxygen would make sense. This was for example observed for *Paracoccus pantotrophus* [56,57].

Steep gradients may also give rise to rapid oxygen dynamics because of slight imbalances in environmental electron donor supply. Further, in many ecosystems the microbial growth rate is low, at least compared to what we are used to in the laboratory. Bacterial generation times may be in the order of days or even weeks. The same would also be true for the lifetime of the enzymes. Under these circumstances it may be simply impossible for denitrifying organisms to rebuild their respiratory chain each time when oxygen comes or goes. And once the denitrification part of the network is in place, it makes no sense not to make use of it, even in the presence of oxygen. Of course, the electrons would still be preferentially channeled to complex IV because of the extra protons translocated, but if the electron flow would exceed the uptake capacity of complex IV, it is likely that the different denitrification enzymes would take care of the overflow.

In such a scenario, denitrification via nitric oxide dismutation would confer an additional advantage, namely that it would require one enzyme less: the combination of canonical denitrification with aerobic respiration requires five different enzymes, whereas nitrite dismutation requires only four. On the other hand, nitric oxide may suffer the disadvantage that the nitric oxide concentration would need to be tightly controled because the chemical reaction of nitric oxide and oxygen has very high affinity.

Aerobic denitrification would be a very favorable process for wastewater treatment, and therefore it has been actively investigated. In the following section, we critically review the published evidence for aerobic denitrification.

## 4. Experimental evidence for aerobic denitrification

As outlined in Section 2, nitrous oxide reductase appears to be the only part of denitrification that is chemically incompatible in the presence of oxygen. The transport of nitrate into the cell was also shown to be affected by oxygen in some cases [51,58], but because many aerobic organisms are perfectly capable of aerobic nitrate import, it is more likely that the observed sensitivity is a specific adaptation for regulatory purposes.

In Section 3 we have argued that denitrification in the presence of excess oxygen does not appear to make sense from a bioenergetic

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perspective but that under dynamic or oxygen limiting conditions one may still expect aerobic denitrification.

Indeed, so far a number of studies have described the observation of aerobic denitrification in the laboratory with pure or mixed cultures (Table 1) (also aerobic nitrifiers have been shown to convert nitrite or hydroxylamine into nitrous oxide, both in the laboratory and in the environment but that process is not addressed further here [59–61]).

It should be noted that in some of the experiments listed in Table 1, nitrogen was measured as the end product. This suggests that different forms of nitrous oxide reductase may exist that are insensitive to oxygen. On the other hand, actively respiring microbes could effectively consume oxygen and keep the oxygen concentration in their surroundings very low, even in the presence of relatively high oxygen concentrations in the headspace or even bulk liquid of the laboratory incubations, especially if the stirring is not sufficiently

**Table 1**
Published evidence for aerobic denitrification.

| Isolate | $O_2$ content (% air saturation) | Lag phase | Products | Reduction rate (M N/d/g protein) | Growth rate ($d^{-1}$) | | | Annotate | Reference |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | $O_2$ | NO*x* | $O_2$ + NO*x* | | |
| 10 Strains belong to genera *Microvirgula*, *Paracoccus*, *Thiobacillus*, *Enterobacter*, *Comamonas* and *Sphingomonas* | ~105.4%[d] | N.A. | $N_2$ ($NO_2^-$) | 0.0031–0.036 | N.A. | N.A. | N.A. | Bacteria were isolated from diverse environment; suspended culture | [62] |
| *Paracoccus denitrificans* NCIB 8944 | 100%[h] | ~5 min | NO*x*↑, $N_2$↓ | 0.045 | N.A. | N.A. | N.A. | Bottle incubation lasted for 5 h; | [63] |
| *Pseudomonas aeruginosa* PA01 | 100%[h] | ~5 min | NO*x*↑, $N_2$↑ | 0.060 | N.A. | N.A. | N.A. | trace $N_2$ produced; suspended | |
| *Pseudomonas aeruginosa* PA0129 | 100%[h] | N.A. | NO*x*↑, $N_2$↑ | 0.073 | N.A. | N.A. | N.A. | culture | |
| *Pseudomonas stutzeri* ATCC 17591 84.60 | 100%[h] | N.A. | NO*x*↑, $N_2$↓ | 0.040 | N.A. | N.A. | N.A. | | |
| *Propionibacterium thoenii* NCDO 568 | 100%[h] | ~5 min | NO*x*↑, $N_2$↑ | 0.065 | N.A. | N.A. | N.A. | | |
| *Pseudomonas* (wild A) | 100%[h] | N.A. | NO*x*↑, $N_2$↓ | 0.0058 | N.A. | N.A. | N.A. | | |
| *Pseudomonas* (wild B) | 100%[h] | N.A. | NO*x*↑, $N_2$↓ | 0.026 | N.A. | N.A. | N.A. | | |
| *Pseudomonas* (wild C) | 100%[h] | N.A. | NO*x*↑, $N_2$↓ | 0.016 | N.A. | N.A. | N.A. | | |
| *Ca. Microvirgula aerodenitrificans* SGLY2 | ~100%[h] | No | NO*x*↑, $N_2$↑ | ≤0.43 | 0.37 | 0.12 | 1.3 | Both adapt and non-adapt suspended culture were used for comparison | [64–66] |
| *Paracoccus pantotrophus* LMD 92.63 | ≤95%[d] | No | $N_2O$, $N_2$↓ | ~$4.12\times10^{-3}$ | N.A. | N.A. | N.A. | $^{15}N$ isotope track; chemostat suspended culture | [67,68] |
| Unknown Strain | 70.5%–84.8%[d] | No | $N_2O$ | 0.0047 M N/d | N.A. | N.A. | N.A. | Bacteria were isolated from an alternating activated sludge system; suspended culture | [69] |
| *Citrobacter diversus* | 12.1%–84.6%[d] | 12 h | NO*x*, $N_2$ | ~0.088 | N.A. | N.A. | N.A. | Suspended culture | [70] |
| *Paracoccus pantotrophus* LMD 37.26 | >80%[d] | No | $N_2O$, $N_2$ | 0.072 | 6.7 | 6.0 | 8.2 | Bacteria were isolated from an sulphide oxidation - nitrate reduction plant; suspended culture | [71–74] |
| *Pseudomonas* sp. LMD 84.60 | >80%[d] | No | N.A. | 0.022 | 2.4 | 3.6 | 9.8 | Simultaneous nitrification and | [75] |
| *A. faecalis* LMD 84.59 | >80%[d] | No | N.A. | N.A. | 4.1 | 1.7 | 6.0 | denitrification were observed | |
| *Ps. Aureofaciens* LMD 37.26 | >80%[d] | No | N.A. | N.A. | 4.6 | 1.7 | 5.0 | | |
| *Aeromonas* (5 isolates) | ≤80%[d] | N.A. | $NO_2^-$ | 0.0072–0.014 | N.A. | N.A. | N.A. | Bacteria were isolated from soil | [76] |
| *Arthrobacter* S2.26 | ≤80%[d] | N.A. | $NO_2^-$ | 0.060 | N.A. | N.A. | N.A. | and sediment samples; suspended | |
| *Moraxella* S2.18 | ≤80%[d] | N.A. | $NO_2^-$ | 0.030 | N.A. | N.A. | N.A. | culture | |
| *Pseudomonas* (16 isolates) | ≤80%[d] | N.A. | $NO_2^-$ | 0.0072–0.072 | N.A. | N.A. | N.A. | | |
| 8 Strains belong to genera *Pseudomonas*, *Delftia*, *Herbaspirillum* and *Comamonas* | 28.8%–79.9%[d] | No | NO*x* | ~0.017 M N/L/d | N.A. | N.A. | N.A. | Suspended culture | [77] |
| *Peseudomonas* sp. yy7 | ~78.6%[d] | 4 h | $N_2$, $N_2O$ | 0.0013 M N/L/d | N.A. | N.A. | 8.2 | Suspended culture | [78] |
| *Pseudomonas putida* AD-21 | 65.5%–78.6%[d] | ~5 h | N.A. | 0.17 ± 0.0095, and 0.56[c] | N.A. | N.A. | ≤3.4 | C/N ratio had a great influence on growth rate, with optimal value of 8; suspended culture | [79] |
| *Ca. Thauera mechernichensis* TL1 | 30%–70%[d] | N.A. | N.A. | 0.023–0.033 | N.A. | N.A. | N.A. | TL1 showed similar trend to *P. denitrificans DSM 2944* under the same condition; suspended culture | [80,81] |
| *Pseudomonas nautical* 617 | 4.78%–33.5%[d] | N.A. | NO*x* | 0.056–0.12 | N.A. | N.A. | N.A. | Kinetic study; suspended culture | [82] |
| Tidal sediments | ≤25.7% | ~1 h | $N_2$ | ≤0.53 M N/$m^3$/d | N.A. | N.A. | N.A. | $^{15}$ N isotope track; aerobic denitrification took place at upper 6 cm in the sediments. | [83] |
| *Pseudomonas aeruginosa* ATCC 9027 | ≤18.3%[d] | N.A. | N.A. | ~0.027 | N.A. | N.A. | N.A. | NAD(P)H contents in the presence of $O_2$ only, $O_2$ and NO*x*, and NO*x* only were lowest, intermediate and highest respectively; suspended culture | [84] |
| *Pseudomonas stutzeri* TR2 | ~16.3%[d] | No | $N_2$, $N_2O$ (trace) | ~$1.26\times10^{-4}$ | N.A. | N.A. | N.A. | Suspended culture | [85] |
| *Pseudomonas* sp. K50 | ~15.9%[d] | No | $N_2$, $N_2O$ (trace) | ~$4.21\times10^{-6}$ | N.A. | N.A. | N.A. | | |
| *Agrobacterium* sp. LAD9 | N.A. | N.A. | N.A. | 0.040 | N.A. | N.A. | 6.9 | Simultaneous nitrification and | [86] |
| *Achromobacter* sp. GAD3 | N.A. | N.A. | N.A. | 0.033 | N.A. | N.A. | 5.5 | denitrification; suspended | |
| *Comamonas* sp. GAD4 | N.A. | N.A. | N.A. | 0.046 | N.A. | N.A. | 10.4 | culture; extracted enzyme showed high Nap activity | |

c: continuous cultivation.
d: dissolved oxygen.
h: oxygen concentration in the headspace.
N.A.: not available.

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rigorous or if the microbes aggregate. For this reason, we closely inspected all methods used in these studies to evaluate whether the conclusion that the claims for the observation of aerobic denitrification are really justified. Table 1 shows whether the reference studies indicated oxygen concentrations, whether oxygen was measured in the bulk or in the headspace and whether the potential presence of anoxic microniches could be ruled out (e.g. by investigating whether the cells were present as aggregates or as suspended cells).

It appears that in most cases, the evidence presented is not sufficient to support the conclusion that denitrification really proceeded in the presence of excess oxygen. It should be noted that the aerobic denitrification rates were much lower than the anaerobic denitrification rates measured for the same strain while aerobic respiration rates remain high. This implies that the major electron flow in the respiratory chain is towards complex IV rather than the NO*x* reductases, and that aerobic respiration is still the preferred pathway for growth. Apparently aerobic denitrification can be an auxiliary pathway next to aerobic respiration.

As is the case for conventional denitrification, aerobic denitrification appears to be taxonomically widespread, at least it was found in Alpha-, Beta- and Gamma-proteobacteria, and this is not surprising as the enzymatic machinery for aerobic and anaerobic denitrification is (probably) the same. Future genome sequencing will resolve this matter.

## 5. A possible scenario for the shared evolutionary origin of aerobic respiration and denitrification

Geochemical studies have shown that oxygenic phototrophy must have evolved in or before the late Archaean, approximately 3 billion years ago [87]. The evolution of aerobic respiration depends on a source of oxygen. If oxygenic phototrophy was the first and only source of oxygen, aerobic respiration must have evolved after the evolution of oxygenic phototrophy. However, other, perhaps relatively small but still significant, chemosynthetic sources may have been available earlier. For example, if a primordial source of chlorate existed, microbial chlorite dismutation could have led to the production of oxygen [88]. Oxygen could also have been generated by catalase-like enzymes acting on peroxides resulting from radioactivity. Finally, it is likely that volcanic eruptions in the primordial atmosphere constituted a significant source of nitric oxide [89]. Via nitric oxide dismutation oxygen could also have been produced. On the other hand, nitric oxide is not a very stable molecule, particularly in the presence of iron. Because in those days the oceans were most likely ferruginous [90–92], nitric oxide or nitrite may be chemically reduced to nitrous oxide and it is unclear to what extent nitric oxide would have been available to biology. It has been argued that copper was not bioavailable in the Archaean because of its precipitation with sulfide [93], but this constraint has been relaxed by the discovery that the oceans were ferruginous and not sulfidic.

In conclusion, geochemical knowledge hardly constrains evolutionary scenarios and is of little help in reconstructing the co-evolution of denitrification and aerobic respiration.

Inferences from biochemistry are slightly more promising: because of the interdependencies of the different complexes/respiratory modules we may make inferences as to what came first and what next. At least we can apply Occam's Razor and infer that those complexes that have the fewest dependencies came first. In this way a simple primordial respiratory chain could have evolved into present, more complicated versions with more (inter)dependencies.

We hypothesize that the simplest respiratory chains do not make use of cytochrome *c* because of its relatively complex maturation system. Respiratory chains depending on the quinol pool only are simpler and still have relatively high bioenergetic efficiency (see Section 2 above). The maturation of cytochrome *c* proteins depends on a dedicated cytochrome *c* maturation system [20–25], on the translocation of proteins across the cytoplasmic membrane and on the presence of complex III or an analogous complex that can relay electrons from the quinol pool to a soluble periplasmic electron carrier like cytochrome *c*. Therefore, the most parsimonious scenario is a respiratory chain consisting of a form of complex I that reduces a primordial quinone followed by reoxidation of the quinone by a terminal electron acceptor. Several forms of complex IV (such as NorZ) could be terminal electron acceptors, do not depend on cytochrome *c* and do not require additional maturation systems. Nitric oxide and oxygen would both be suitable substrates for such a complex because also current enzymes are not completely specific (see above). Initially, there may not have been a need for energy conservation: the primordial respiratory chain may have simply served as a sink for NADH for fermenting microorganisms and for the removal of oxygen to prevent radical chemistry. This strategy is also followed by current fermenters that reduce nitrate to ammonium or sulfur to sulfide.

Once this simple form of complex IV evolved, a driving force was established for the evolution of its proton translocation channels, leading to improved bioenergetics. If nitric oxide was the primordial substrate, nitric oxide dismutation could have produced the same final result: proton translocation.

Nitrous oxide would be a chemically stable end-product and would have accumulated in the biosphere, a driving force for the evolution of nitrous oxide reductase so resulted. Again, it is likely that the quinol-dependent integral membrane form was the first protein that evolved [94].

It is likely that nitrate and nitrite consuming enzymes evolved much later, only after the evolution of cytochrome *c* and nitrification. Production of nitrite and nitrate in nitrification depends on cytochrome *c*.

In conclusion, from a biochemical perspective it is most likely that the integration of both denitrification and aerobic respiration into a respiratory chain started with the evolution of a primordial form of complex IV that reduced both oxygen and nitric oxide or even interconverted these two molecules.

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