

Mitigating release of the potent greenhouse gas N₂O from the nitrogen cycle – could enzymic regulation hold the key?

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When faced with a shortage of oxygen, many bacterial species use nitrate to support respiration via the process of denitrification. This takes place extensively in nitrogen-rich soils and generates the gaseous products nitric oxide (NO), nitrous oxide (N₂O) and dinitrogen (N₂). The denitrifying bacteria protect themselves from the endogenous cytotoxic NO produced by converting it to N₂O, which can be released into the atmosphere. However, N₂O is a potent greenhouse gas and hence the activity of the enzyme that breaks down N₂O has a crucial role in restricting its atmospheric levels. Here, we review the current understanding of the process by which N₂O is produced and destroyed and discuss the potential for feeding this into new approaches for combating N₂O release.

Introduction

Nitrous oxide (N₂O), commonly known as laughing gas, is a colourless and non-toxic gas, discovered over 200 years ago, that has since been used as an anaesthetic and fuel additive. Recently it has received attention because it is a powerful greenhouse gas that can persist for up to 150 years while it is slowly broken down in the stratosphere [1–3]. Although N₂O only accounts for around 0.03% of total greenhouse gas emissions, it has a 300-fold greater potential for global warming effects, based on its radiative capacity compared with that of carbon dioxide (CO₂) [4]. Hence, when the actual impact of individual greenhouse gases on global warming is expressed in terms of the Intergovernmental Panel on Climate Change approved unit of CO₂ equivalents, N₂O accounts for ~10% of total emissions [4]. More than two-thirds of these come from bacterial and fungal denitrification and nitrification processes in soils (Box 1) [2,5]. This contribution has been exacerbated through the intensification of agriculture, the so-called ‘green revolution’, which has increased the presence of nitrogen (N) in soil through the application, since the early 1900 s, of synthetic nitrogen-based fertilizers. Intensive pastoral farming, in which animal waste is returned to soils [2] and wastewater treatment further

contribute to increasing N₂O emissions [1,2]. The cumulative effect over the past century has been an ~20% increase in atmospheric N₂O concentration [2], which is further increasing at a rate of 0.2%–0.3% per year [5]. The 1997 Kyoto Protocol set emission limitations and reduction obligations with respect to a basket of six gases, including N₂O, on its signatories. Since 1997, many non-biological emissions, for example those associated with transport industry, have been lowered but emissions from agriculture remain essentially unchanged. The Kyoto Protocol expires in 2012 and it is important that its successor is able to address fully the issue of soil-derived N₂O emissions. Here, we review recent progress in understanding the molecular basis for generating and consuming N₂O through bacterial and fungal denitrification processes and reflect on how this could feed into the development of strategies for mitigating release.

Denitrification and biological N₂O emission

Many bacteria can grow either in the presence or absence of oxygen (O₂). O₂ is required for aerobic respiration, which confers enormous energetic benefits on bacteria by allowing the complete oxidation of a growth substrate and the concomitant conservation of the large amounts of energy released. However, when faced with a shortage of O₂, some bacteria, exemplified by the soil bacterium *Paracoccus denitrificans*, can switch to respiring nitrate (NO₃⁻) in a process known as denitrification (Figure 1 and Box 2) during which NO₃⁻ is converted, via nitrite (NO₂⁻), to the gases nitric oxide (NO) and N₂O, and then to the inert gas dinitrogen (N₂). Four enzymes are required sequentially to reduce NO₃⁻ ion to N₂. Each enzyme uses a redox active metal cofactor, such as molybdenum for NO₃⁻ reduction, iron or copper for NO₂⁻ reduction, iron for NO reduction, and copper for N₂O reduction (Figure 1). Key sensors for effecting the change from O₂ respiration to denitrification are DNA-binding proteins that regulate transcription of genes in response to environmental factors, such as an O₂-sensitive DNA-binding protein, called FNR (fumarate nitrate reduction regulatory protein), that measures the declining level of O₂ using an iron–sulfur cluster, as well homologues of this protein, NNR (nitrite

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Box 1. The nitrogen cycle

Nitrogen (N) is essential for all organisms and on average it accounts for up to 6.25% of their dry mass and ~78% of the atmosphere [83,84]. The N cycle involves several redox reactions performed by different enzymes and organisms whereby N goes through a variety of oxidative and reduced states ranging from +5 (NO_3^-) to -3 (NH_4^+) [83], as illustrated in Figure 1. Many of these reactions are carried out in bacteria, archaea and some fungi. A phylogenetically wide range of species use the intermediates of the N cycle as electron acceptors in anaerobic respiration. Assimilatory NO_3^- reduction occurs in plants and prokaryotes is where NO_3^- is reduced to NH_4^+ via NO_2^- [83]. Generally, NH_4^+ is used for the synthesis of glutamine, which is then the N-donor for the synthesis of other amino acids and heterocyclic N-compounds and signifies the importance of N for organisms [83].

One of the main pathways in the N cycle is denitrification, which refers to the dissimilatory reduction of one or both ionic nitrogen oxides, NO_3^- and NO_2^- , to the gaseous oxides, NO and N_2O , which might be further reduced to N_2 . Denitrification is a very important process in many ways; it is a major problem in farming because it decreases the effectiveness of fertilizers due to the removal of NO_3^- [85]. The removal of NO_3^- is vital in wastewater treatment because NO_3^- leakage can lead to eutrophication [85]. In addition, denitrification can be a contributor to the greenhouse effect due to the intermediates formed, N_2O and NO [1,39,85]. NO_3^- can also be reduced to NH_4^+ via NO_2^- ; this process, known as $\text{NO}_3^-/\text{NO}_2^-$ ammonification, typically occurs in Enterobacteriaceae [83].

There are a variety of organisms that can oxidize NH_4^+ or NO_2^- to attain energy, known as nitrification. This process has been found to occur in autotrophic and heterotrophic nitrifying organisms, which thrive in soils [83]. Anammox (anaerobic ammonium oxidation) is a novel process whereby NH_4^+ is oxidized to NO_2^- , which is in turn used as an electron acceptor, resulting in the final product being N_2 . This process is of great interest, especially in wastewater treatment, because it is a means of reducing nitrite in wastewater, which can

have major implications on the environment. The step which completes this complex cycle is N fixation, the reduction of N_2 to NH_4^+ [83]. This process occurs in prokaryotes, some of which form a symbiosis with plants.

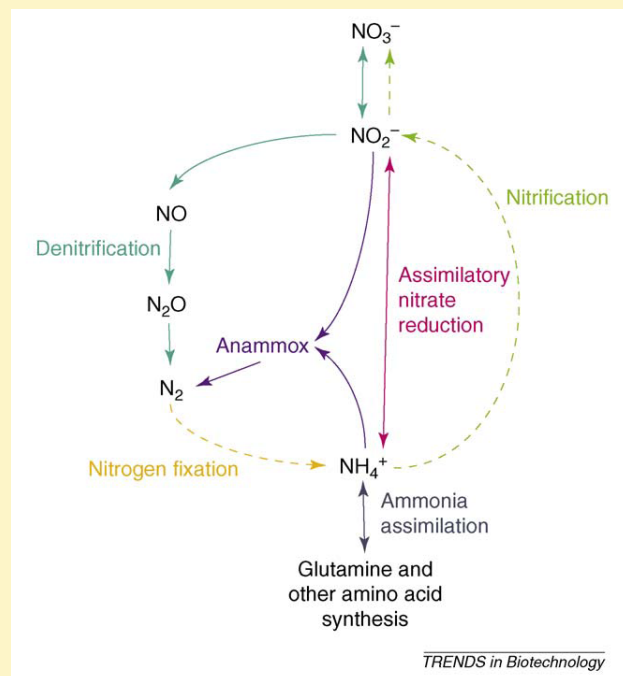


Figure 1. Schematic illustration of the nitrogen cycle.

and nitric oxide reduction regulatory protein) and NarR (nitrate reduction regulatory protein), which sense NO, NO_2^- and NO_3^- [6–11]. NO is a potent cytotoxin, and bacteria deficient in nitric oxide reductase (Nor) cannot grow through denitrification because they are killed by this toxic intermediate [12,13]. Thus, ideally, the NO-generating and NO-consuming reactions of denitrification should optimally function in a concerted fashion to form the relatively benign N_2O . If the bioenergetics of denitrification are considered (see Box 2), there is little disadvantage for the cell in failing to perform the final step of N_2O reduction. Indeed, the fact that so much N_2O is produced from soils carrying out bacterial denitrification implies that the enzyme nitrous oxide reductase (N_2OR) or the bacterial population as a whole do not always carry out the final step either efficiently or in synchrony with upstream parts of the pathway. Furthermore, some denitrifying bacteria, such as *Agrobacterium tumefaciens* [14], do not even have the gene for N_2OR . Likewise, denitrifying fungi also lack N_2OR [15]. Efforts to improve the management of N_2O emission, which the Kyoto Protocol's successor will undoubtedly require, will benefit from a better understanding of the factors that influence the net production of this gas by denitrifying bacteria, both at the enzyme and soil population level.

Producing and reducing N₂O

N_2O is produced by the sequential action of the NO_3^- , NO_2^- and NO reductases (Figure 1 and Box 2). Because some of the denitrification enzymes have active sites

that lie towards the outside (Nor) or are actually outside (periplasmic nitrate reductase [Nap] and nitrite reductase [Nir]) of the cytoplasmic membrane (see Figure 1), their operations are likely to be sensitive to the environmental pH, and typically these enzymes are most active at pH of 7 or below [16–19]. The NO_3^- , NO_2^- and NO reductases are generally tolerant of O_2 ; indeed, both Nor and the iron-containing cytochrome cd_1 Nir can catalyse the four-electron reduction of O_2 to water. It is notable that some nitrifying bacteria that oxidize ammonia (NH_3) to NO_2^- also have some of the denitrifying enzymes that lead to N_2O production. For example *Nitrosomonas europaea* has a copper-dependent Nir and a Nor, but no N_2OR [15,20–28]. Hence it is a potential producer, not a consumer, of N_2O . Although not a major focus of this article, bacteria that reduce NO_3^- to ammonium (NH_4^+) (so-called ammonifiers, see Box 1) can also produce N_2O as a by-product, but most do not have an enzyme that can consume it. The contribution that ammonifying bacteria make to global N_2O emissions is not known but should be addressed. In fungi, the enzymes that reduce NO_3^- and NO_2^- have similarities to those of denitrifying bacteria [15], but the enzyme that detoxifies NO to produce N_2O is a cytoplasmic enzyme [28]. However, fungi also are thought not to have an N_2OR and as such are also net emitters of the greenhouse gas.

There are several enzymatic and microbial routes to N_2O production. However, the bacterial N_2OR , is the only known enzyme capable of reducing N_2O to N_2 . The reaction, shown in Equation 1, is highly favoured thermody-

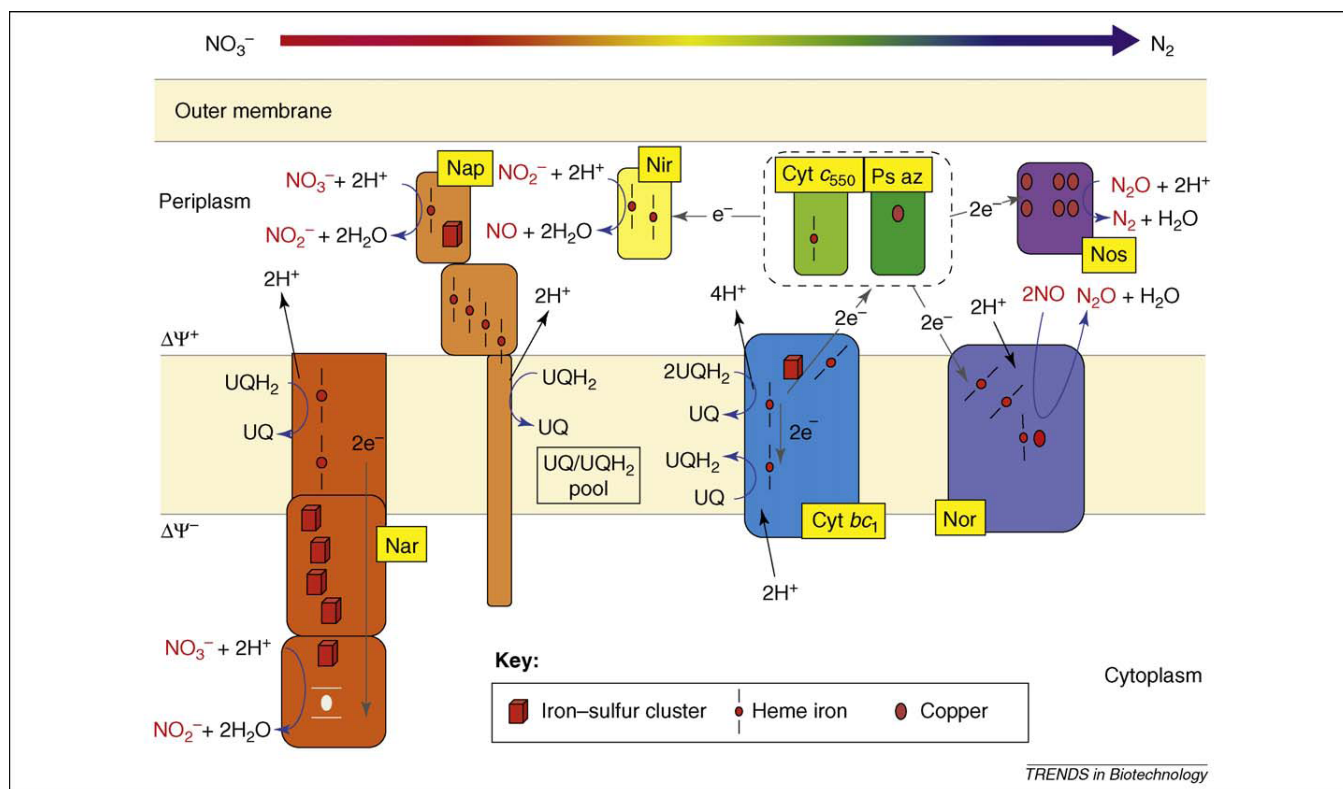
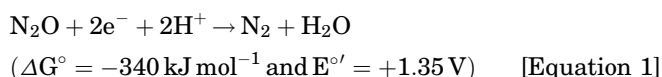


Figure 1. Schematic illustration of denitrification enzymes, their cellular location and dependency on metal cofactors. The pathway shown is representative of that found in many Gram-negative bacteria, such as *P. denitrificans*. Note, however, that some denitrifying bacteria have a copper-containing, rather than heme-containing, nitrite reductase. The denitrification leads to the net movement of protons from the cytoplasm to the periplasm, which creates a proton motive force in which the membrane potential ($\Delta\psi$) is positive on the outside of the inner membrane. The electrons for each reaction originate from the ubiquinol pool (UQH₂), which is replenished by electron input from the oxidation of organic carbon or inorganic electron donors. Abbreviations: Nar, membrane-bound nitrate reductase; Nap, periplasmic nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase system; Ps az, pseudoazurin; Cyt *bc*₁, cytochrome *bc*₁; Cyt *c*₅₅₀, cytochrome *c*₅₅₀. As the diagram flows from left to right, the electron acceptor becomes progressively more reduced with NO_3^- being reduced to N_2 gas.

namically (indicated by the large negative value of the free energy, ΔG°).



N_2O is a powerful oxidant with respect to N_2 , as shown by the high positive redox potential at pH 7, $E^{\circ'} = +1.35 \text{ V}$ vs NHE. However, N_2O binds very poorly to metal ions and is difficult to activate towards its reduction. Biology has overcome this problem by evolving in N_2OR a unique

Box 2. The bioenergetics and enzymology of denitrification

The structures of the key enzymes that reduce nitrate to nitrite and thence to nitric oxide have been determined over the course of the past decade or so [7]. These include two distinct types of nitrate reductase, both of which are molybdo/heme/iron-sulfur cluster enzymes, and two types of nitrite reductase – one a copper enzyme and the other a heme iron enzyme (see Figure 1 in the main text). The reductive condensation of two molecules of NO to form N_2O is catalysed by nitric oxide reductase (Nor). Three types of respiratory Nors have been reported in Eubacteria [86,87], but the best studied is the cytochrome-*c*-dependent Nor from *Paracoccus denitrificans*, which is purified as a two-subunit complex, known as NorBC [86]. The NorC subunit is a mono-heme *c*-type cytochrome that possesses an N-terminal transmembrane helix that anchors the heme domain to the periplasmic face of the cytoplasmic membrane and serves as the immediate electron acceptor for two soluble periplasmic electron donors: pseudoazurin and cytochrome *c*₅₅₀. The catalytic subunit, NorB, comprises 12 transmembrane helices that bind three metal centres: a magnetically isolated bis-histidine-coordinated low-spin heme and an active site, comprising a high-spin heme and an iron ion (Fe_B) site magnetically coupled to form a dinuclear centre [88]. NorB shares the same overall architecture with heme copper oxidases [89,90], and homology modelling shows that the heme: Fe_B dinuclear centre, at which N_2O is formed, is buried in the lipid bilayer. The

modelling also envisions a pathway to conduct protons from the periplasm to the buried active site [13,91,92].

The denitrification enzymes are associated with the energy conserving membrane of bacteria. As each intermediate is reduced, protons can be moved across the membrane to generate a proton-motive force. This is used to drive the synthesis of the fuel ATP, with an enzyme ATP synthase consuming the proton-motive force to make ATP. In many ways the system is similar to the oxygen-consuming respiratory system of human mitochondria, but with oxidized nitrogen species rather than oxygen terminating the electron transport chain. The complete reduction of nitrate to dinitrogen gas requires ten electrons. The reduction to N_2O requires eight electrons. If these electrons originate from NADH, then there is an associated translocation of 30 protons across the energy conserving cytoplasmic membrane that creates a proton-motive force to drive ATP synthesis (~3.3 protons/ATP). If denitrification does not proceed to completion, then this becomes 24 protons. Thus there is only an ~20% difference in efficiency and thus the lack of N_2O reduction makes very little difference to the bioenergetics of the bacterium. This is perhaps reflected by several denitrifying bacteria that can reduce nitrate to N_2O but do not have a gene encoding for a N_2O reductase, so that denitrification terminates in N_2O release.

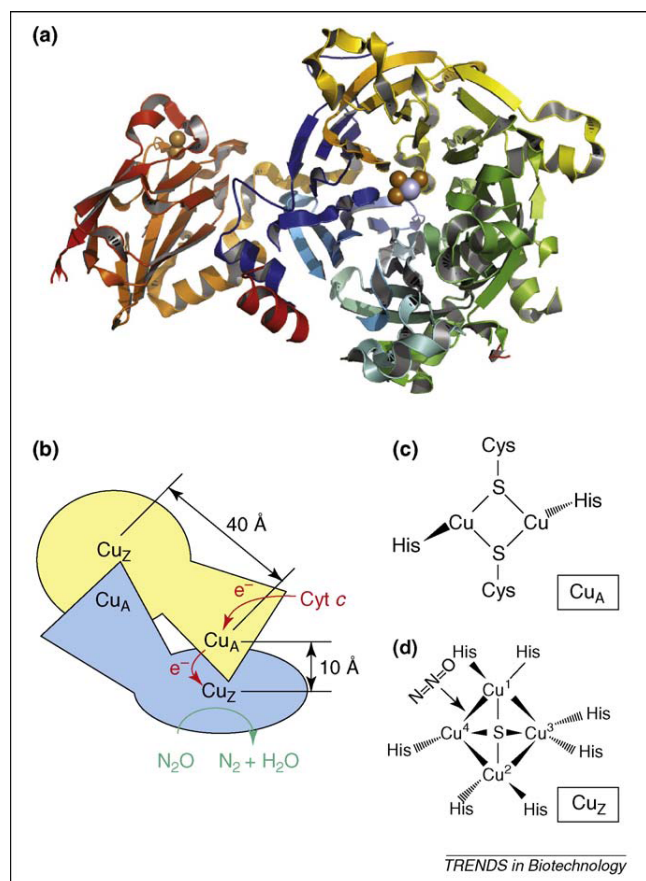


Figure 2. The molecular structure of nitrous oxide reductase (N₂OR). (a) The structure of N₂OR. X-ray crystal structure of a monomer of the *P. denitrificans* enzyme showing two domains, a β -barrel and a seven-bladed β -propeller, each binding one copper cluster. Drawn using PyMol from coordinates found in Protein Data Bank 1FWX [30]. (b) Schematic illustration of the dimeric form of N₂OR illustrating the path of electrons from the donor cytochrome *c* via Cu_A to Cu_Z. The Cu_A and Cu_Z centres are ~40 Å apart, which is too far for electron transfer between them to proceed at a useful rate. The head-to-tail alignment of the protomers in the homodimer brings the Cu_A centre of one monomer within ~10–12 Å of the catalytic Cu_Z site in the other monomer. Thus electron transfer must take place across the interface, implying that the dimer is the catalytically active unit (adapted from Refs [29–31,33,56–59]). (c) Molecular structure of Cu_A, showing the binuclear thiol-bridged copper centre that binds to the protein via two histidine ligands and undergoes a one-electron redox change, between [Cu^I–Cu^I] and [Cu^I–Cu^{II}]. (d) Molecular structure of the catalytic centre, Cu_Z [15–17,19,33,56–60], a copper–sulfur cluster [Cu₄S]^{III+}. Each copper ion is ligated by one or two histidine residues, making seven in total. [Cu₄S]^{III+} can undergo a two-electron redox cycle from the mixed-valence state [Cu₂(II)Cu₂(I)S] to the all-copper(I) state [Cu₄(I)S] [61,62]. The linear molecule N₂O is thought to bind along one Cu–Cu edge of the cluster as shown [63].

multi-copper-sulfide centre, so-called Cu_Z, that is capable of catalysing the activation and two-electron reduction of N₂O gas, as illustrated in Figure 2.

N₂OR is a soluble enzyme located in the bacterial periplasm. The electron donors to *P. denitrificans* N₂OR *in vivo* are cytochrome *c* and the cupredoxin pseudoazurin (see Figure 1). Crystal structures of the enzyme are now available from three bacterial species: *Pseudomonas nautica*, *P. denitrificans* and *Achromobacter cycloclastes* (Figure 2) [29–31]. All are virtually identical and show that N₂OR is a homodimer with each monomer binding six copper ions in the form of two copper clusters, a di-nuclear electron transfer and storage site, Cu_A and the tetra-nuclear cluster [Cu₄S], Cu_Z. N₂OR can exist in several forms possessing very different enzymatic activities towards N₂O reduction. These

might arise from changes at the catalytic site [Cu₄S], although it cannot be excluded that other factors might also have a role, such as the electron transfer rate from Cu_A to Cu_Z that takes place across the protein dimer interface (Figure 2). However, it is clear that Cu_Z is inactivated by the denitrification intermediate NO and by O₂, both of which could impact on its activity in the field [32]. Exposure to O₂ results in cofactor Cu_Z apparently becoming trapped in a redox-inactive form of the state [Cu₄S]³⁺ [33]. This behaviour is reminiscent of some other metallo-enzymes that are designed to reduce small gaseous substrates at low potentials, such as hydrogenase, which reduces protons or oxidizes hydrogen gas. These enzymes seem to have strategies to protect their highly activated catalytic sites from O₂ damage or to even allow enzyme turnover in the presence of limited amounts of O₂. Not only can transient exposure to O₂ irreversibly damage the catalytic site, but the generation of reduction products of O₂, such as superoxide ions, can also be toxic to the cell. To be able to reduce the inert substrate N₂O, the catalytic site will inevitably also be highly reactive towards O₂. Therefore, a means of shutting down the enzyme by forming a redox-inactive state of the catalytic centre using O₂ itself could have a functional advantage. However, the activation and the deactivation of N₂OR within bacterial cells that are carrying out denitrification could also be of consequence for the emission of N₂O from a bacterial population. If N₂OR were to be deactivated within a cell by, say, transient exposure to O₂, then N₂O will not be reduced to N₂ and N₂O emissions into the environment will inevitably rise. Evidence that this might occur will be discussed later.

Like the denitrification enzymes that lead to synthesis of N₂O, the activity of N₂OR is also sensitive to pH [34–36]. *In vitro*, the active form of the enzyme that is generated using methylviologen as an electron donor is more active at pH > 7. This contrasts with the general pattern observed with NO₃[−], NO₂[−] and NO reductases, which are generally more active at pH < 7. This could then explain field data that often show that low pH in soils drives the reduction of NO₃[−] to N₂O gas rather than to N₂. However, the pH dependency of the enzyme *in vitro* seems to be dependent on the nature of the electron donor, and this effect requires further investigation [34,35]. In addition, a recent significant observation of the effect of pH on N₂O release from a wastewater reactor has suggested that free nitrous acid, generated from NO₂[−] at low pH, inhibits N₂OR, which implies that the effect of pH on N₂O reduction could, at least in part, be indirect [37].

The active dimeric form of N₂OR contains twelve copper ions [30,31], which places a high demand on the bacterium for an adequate supply of copper from its environment. Should the copper supply fail, the biosynthesis of the enzyme will be incomplete and the resulting enzyme will be inactive. Indeed, it has been shown that removing copper ions from a bacterial culture while it is carrying out denitrification causes N₂O emissions to rise, whereas adding copper to the growth medium causes N₂O emissions to drop and N₂ emissions to increase [38,39]. There are other enzymes in bacteria that are dependent on copper, such as cupredoxin electron transfer proteins, heme-copper oxidases and superoxide dismutases, but

in all these cases, alternative non copper-containing enzymes exist that can perform the same functions. Indeed, in the case of the methane mono-oxygenases of the methanotrophs that consume the potent greenhouse gas methane, iron-dependent and copper-dependent forms are differentially regulated in response to copper availability [40]. However, there is no alternative non-copper enzyme to N₂OR for reducing N₂O to N₂. Thus, although it is an area that requires more extensive study, a copper-deficient denitrifying bacterial community is expected to remain viable, but will be releasing N₂O. The question then arises of whether denitrifying bacteria have the capacity to sense copper and to regulate N₂OR synthesis in a copper-dependent manner. This has not yet been assessed, but it is intriguing that the structural gene encoding N₂OR, *nosZ*, is co-transcribed with *nosR*, which encodes a transcriptional regulatory subunit that has a cytoplasmic cysteine-rich cluster and that could potentially be a metal-binding site [41]. However, the biosynthesis and assembly of the enzyme requires the products of several *nos* genes, including a putative transporter encoded by *nosDFY*, and the Cu chaperone NosL for biogenesis of the metal centre [1,41,42]. NosR is a membrane protein with large soluble domains that contact both the periplasm and cytoplasm [42] and is involved in transcriptional regulation of *nosZ* [41]. The NosL gene product is thought to be an outer membrane protein [1]. Homologous genes have been found in other denitrifiers, such as *Pseudomonas aeruginosa* [1]. Little is known about the structures or functions of any of these gene products or their roles in the assembly of the copper cofactors of N₂OR. The mechanism of uptake of copper into denitrifying bacteria is also obscure. An understanding of these properties is likely to be key to a full appreciation of the role of environmental copper in N₂O emissions.

Modelling the production and consumption of a potent greenhouse gas

Simple sequential models for denitrification can be formulated that involve only the K_M and V_{max} of each enzyme for its nitrogenous substrate. In such models, there is no significant accumulation of an intermediate if the K_M for the substrate is low and if the rate of each step is faster than that which precedes it. In practice, this is rarely the case because each enzyme can have a different pH dependency, be differentially susceptible to inhibition by other environmental variables, such as O₂ or the availability of copper ions (as we have already discussed), or might be in competition with each other for the same substrate. This latter point arises because each denitrifying enzyme requires electrons from a common source, the ubiquinol (UQH₂) pool of the respiratory electron transport chain (Figure 3), and competition for electrons in denitrification has been used in an assessment of the impact of pH on denitrification [43]. Experiments revealed little accumulation of N₂O at pH 8.5, an observation that is consistent with a simple sequential model. By contrast, at pH 5.5, both NO₂⁻ and N₂O accumulated transiently up to 300 μM. A kinetic model to describe the experimental data includes the use of two-substrate kinetics with an estimate of V_{max} and two K_M values for each reaction (for the electron donor and electron acceptor) and two branch points at the level of the quinol pool and cytochrome *c* pool [43] (Figure 3). Branched models reflect the modular nature of the electron transport chain but must also be sufficiently robust to be applied to denitrification when additional environmental complexities arise [44]. For example, aerobic denitrification in response to metabolism of highly reduced carbon substrates or the inactivation of N₂OR by O₂, free nitrous acid or NO. In the latter case, the kinetics of enzyme synthesis and repair require measure-

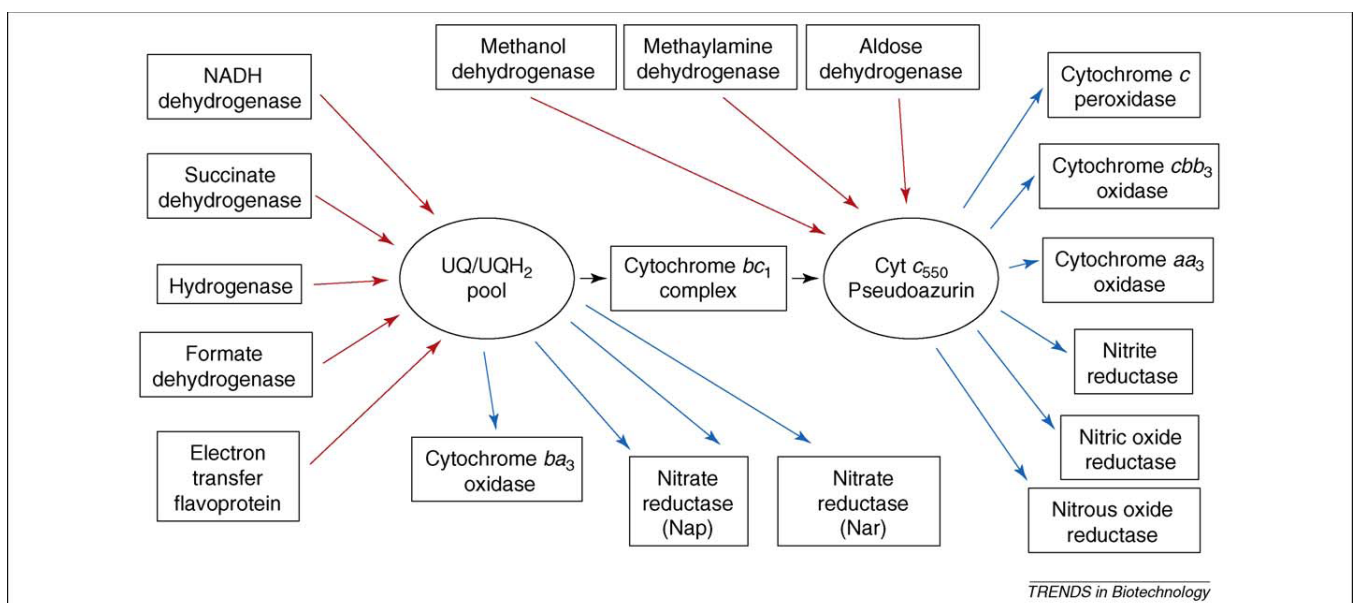


Figure 3. An illustrative branched electron transport network for *P. denitrificans*. The diagram illustrates the branched nature and modular arrangement of the network. The ubiquinone/ubiquinol (UQ/UQH₂) pool and the cytochrome *c*₅₅₀ (Cyt *c*₅₅₀/pseudoazurin) pools are key branch points into which a number of electron donors (red arrows) flow and which can be drawn out by several electron acceptors (blue arrows) [64]. It is notable that different denitrification enzymes branch from different modules, which needs to be accounted for in models. Note that Nap and Nar represent the different nitrate reductases illustrated in Figure 1.

Review

ment of rates of transcription, translation and assembly of the proteins involved in denitrification. The overall coordination of regulation and biosynthesis is not yet sufficiently well understood to allow it to be modelled. Thus studies are needed of time-series of fluxes and expression data using modelling methods such as hierarchical and metabolic regulation analysis [45]. This would enable dissection of the different levels (or hierarchies) of regulation that might underlie some of the changes in N_2O flux.

Mitigating N_2O emissions by enhancing the transformation of N_2O to N_2

Globally, we stand on the brink of some major opportunities in agriculture and food production for lowering the production of greenhouse gases, such as N_2O . For example, the current interest in production of crops for second-generation biofuels necessitates understanding of the environmental controls and consequences of intensive crop production [46]. Little is known of the direct and indirect effects on greenhouse gases of bioenergy crop and biofuel production, but there are estimates indicating that N_2O is the largest greenhouse gas source in these systems [47]. Surprisingly little is known about this regulation, particularly of N_2O reduction by N_2OR , either at the level of the gene or the protein, yet this enzyme should surely be central to any strategy for the mitigation of N_2O emission. Direct measurements of N_2O reduction in the field are scarce, in part due to difficulties of quantifying N_2 production against a large background atmospheric concentration. Most existing knowledge is inferred from

experiments in controlled environments utilizing stable isotope approaches or N_2 -free headspaces, or from field experiments involving the application of 10 kPa acetylene as an inhibitor of N_2OR . However, the latter can lead to inaccuracies, not least because this inhibitor can be used as a substrate for denitrification if carbon is limiting [20,22]. The primary drivers of N_2O production and reduction in the field are thought to be O_2 partial pressure, nitrogen and carbon availability, and soil pH [24]. The N_2O mole fraction, that is, the molar ratio between N_2O and (N_2O+N_2) , represents the relative proportion of nitrogen lost as N_2 and N_2O during denitrification and is generally reported to range from 0.05 to 1 [26], [23]. This mole fraction increases with low pH [25] but decreases after addition of carbon [27] and a decrease in O_2 concentration [48]. It can also be high after addition of NO_3^- fertilizers because at concentrations of $>10 \text{ mg } NO_3^- \text{ g}^{-1}$ soil NO_3^- is preferred over N_2O as an electron acceptor [21].

The ability to denitrify is phylogenetically diverse, and recent developments in techniques for quantifying N_2O production by denitrification are showing its occurrence to be more widespread than previously thought. Thus, it is unlikely that it will ever be possible to develop farming practices that completely eliminate denitrifier- N_2O emission from agriculture. Here, we propose that a more reliable approach to mitigating N_2O emissions would be to translate emerging knowledge of the enzymology of denitrification into protocols designed to manipulate the physiology of denitrifying bacteria so that the extent of reduction of N_2O to N_2 is not constrained but, instead,

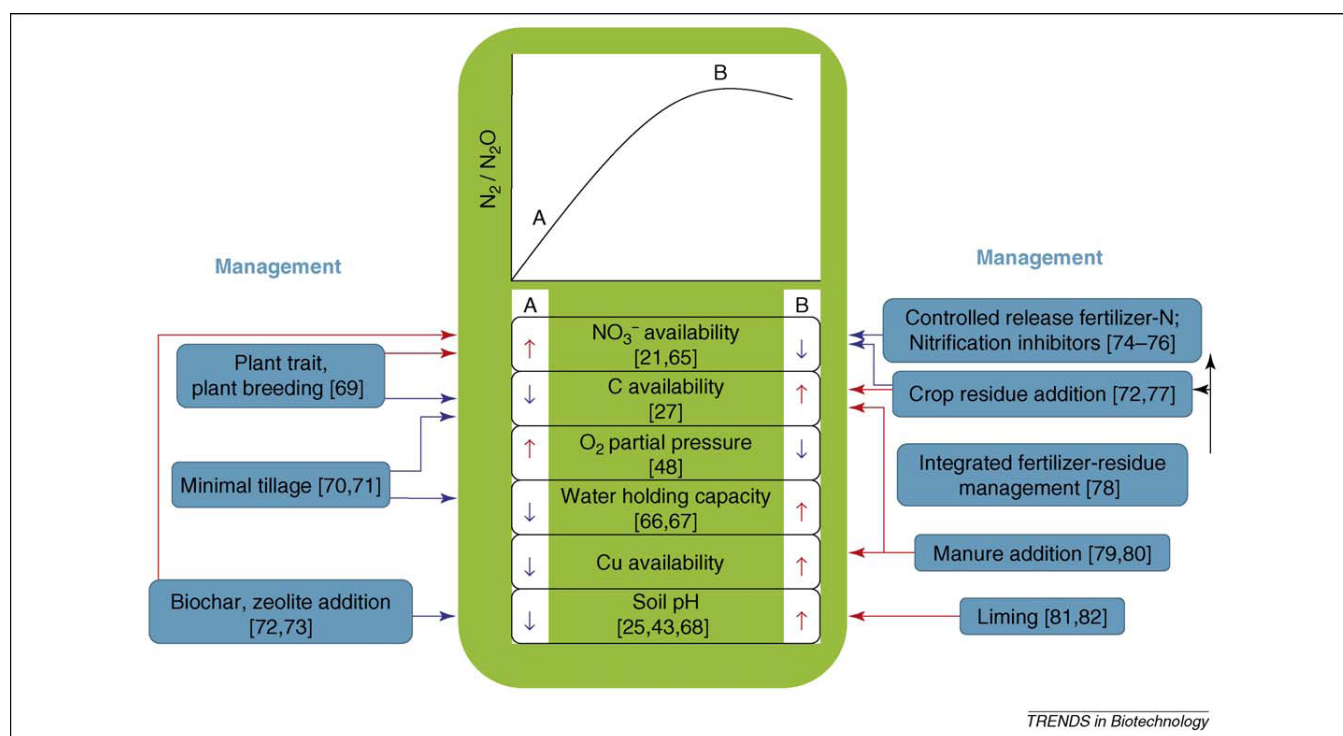


Figure 4. Factors that can affect the ratio of N_2/N_2O emitted from agricultural soils. The diagram shows a selection of proposed management options by which soil conditions could be manipulated to either lower emission of N_2O , or to increase its reduction to N_2 . This highlights areas in which understanding of the regulation of N_2O reduction can be taken forward into practical management options for mitigating N_2O emission from soil. Here we indicate the potential response of N_2/N_2O ('A' corresponds to low N_2/N_2O and 'B' to high N_2/N_2O) to increases or decreases of these factors, although the specific effects of these management options on factors central to driving N_2O reduction to N_2 are unknown. It should be noted that there are also likely to be significant interactions between factors. Trends presented are informed from data in Refs [21,25,27,43,48,65–82], which also contain detailed descriptions of these management practices.

increased. In practice, this might be possible through plant breeding to manipulate denitrification through inputs into the plant rhizosphere, thereby changing the composition of plant-derived carbon flow or nitrogen uptake demand, or through crop spacing, tillage or integrated inorganic fertilizer, residue and soil organic matter (SOM) management (Figure 4). Breeding for plant-release of biological nitrification inhibitors that block the ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) pathways in ammonia-oxidizing bacteria offers the promise for manipulating soil nitrogen concentrations [49], and hence the soil denitrification potential. However, the effects on N₂O production are unknown. Such opportunities for managing N₂O emissions need to be considered in light of effects on soil carbon levels and chemistry, not only because of the other key greenhouse gases – CO₂ and methane – but also because of the important balance between fertilizer application increasing carbon sequestration through greater biomass production versus the undesirable alternative consequence of increased N₂O emission [50].

Considering what we can learn from enzymology to drive N₂O reduction to N₂, our view is that N₂O gas emissions from bacterial denitrification processes in soils arise largely from a failure of the final step in the pathway, the reduction of N₂O to N₂. We believe that there could be at least two reasons for this: first, the biosynthesis and/or assembly of N₂OR is incomplete owing to the lack of sufficient copper to provide a full complement of copper cofactors Cu_A and Cu_Z. This could arise in several ways. Copper in the soil could become limiting because of the high density of bacterial cells due to, for example, the application of high levels of NO₃⁻ fertilizer stimulating rapid bacterial growth. Alternatively, the nature of the soil, such as a high humic acid content, might sequester free copper ions, with strong adsorption at pH ≥ 7, causing their bioavailability to be low. It could also be that low levels of available copper cause biosynthesis of the N₂OR apo-protein to be shut down completely. We therefore suggest that there is considerable potential to enhance N₂O reduction in soil, either through using SOM management or liming as primary controls of copper availability (Figure 4). It should be noted that copper-limitation will directly impact only on N₂O release because the limitation of iron or molybdenum, the other ions that form cofactors in denitrification enzymes (see Figure 1 and Box 1), would lead to inhibition of flux at early stages in the process, for example NO₃⁻ reduction, so that N₂O would not be formed. Of some interest also will be the effect of copper limitation on denitrifying communities in which the copper-type rather than the heme-iron type of Nir predominates. If NO₂⁻ is dysfunctional in such bacteria, NO₂⁻ could be the major end product of denitrification, which could stimulate nitrification reactions (Box 1).

The second feature to which we drew attention earlier is the possibility that N₂OR can be temporarily inactivated by sudden exposure to even low levels of O₂ while the other enzymes of denitrification would continue to function. This has been illustrated in recent work in which the effect of O₂ on NO₂⁻ dependent denitrification and the emission of NO, N₂O and N₂ was investigated in soil-extracted bacteria

[48]. In aerobic-adapted cultures, N₂O was only detected at ≤ 80 μM O₂ but, once O₂ was completely depleted, the denitrification rate was three orders of magnitude higher and N₂ was the primary product. However, when cultures were re-exposed to O₂, they continued to denitrify at up to 55% of the rates observed during the earlier anoxic phase, but the major product was N₂O. However, the N₂O reduction activity recovered as O₂ was depleted after this re-exposure period. These results suggest that pre-synthesis of the denitrifying proteome might result in significant subsequent 'aerobic' denitrification, but that this only proceeds as far as N₂O owing to the O₂-dependent inactivation of N₂OR. Enzyme repair or *de novo* synthesis might then enable the culture to recover the capacity for N₂O reduction. This study illustrates how understanding the molecular properties of N₂OR and its mechanisms of synthesis and activation could in turn help in understanding how short anoxic spells cause N₂O production. For example, transient flooding during rainfall could lead to subsequent increases in N₂O release by stimulating denitrification and hence N₂O production, because N₂OR will be inhibited by O₂ once the water levels have dropped. Understanding such regulation will be central to optimizing organic matter, pH or N management practices for increasing N₂O reduction to N₂ and will influence the timing of the addition of organic matter and the management of soil pH or nitrogen levels.

Finally, another way of enhancing conversion of N₂O to N₂ would be to use plants to 'scrub' the emissions by expressing bacterial N₂OR in plants. At present this would be an ambitious genetic engineering project and clearly there would be societal opinions to consider with respect to acceptance of genetic engineering strategies by the general public. Because N₂OR is confined to the bacterial and archaeal worlds, it would have to be coupled to a respiratory electron transport pathway, such as that of plant root mitochondria. However, there would also be the difficulty of correctly assembling the Cu_Z site. This is a challenge even in heterologous expression in bacteria. Recently, the synthesis of *Geobacillus thermodenitrificans* N₂OR [36] was attempted in *Escherichia coli* but yielded a stoichiometry of only 2.5 Cu/monomer rather than the required 6. It is noteworthy that nature already confers on some plant roots the capacity for N₂O reduction through symbiosis. Many legume root symbionts have the capacity for N₂O reduction, for example the soybean symbiont *Bradyrhizobium japonicum* [51,52]. In addition to conferring the ability to fix N, this bacterium also provides the ability to reduce N₂O. However, the impact of cropping leguminous plants with N₂O-reducing root symbionts on soil N₂O production has not yet been assessed.

Concluding remarks

In this article we have reviewed the denitrification process in the nitrogen cycle that leads to the production and consumption of the potent greenhouse gas N₂O and described the biochemical properties of the N₂OR enzyme, which catalyses the major route for biological N₂O consumption on the planet. Because N₂OR is a copper enzyme, biological N₂O consumption is an obligatory copper-dependent process. We have considered whether understanding

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Box 3. Outstanding questions to be addressed experimentally

- What is the pH dependency of N₂OR *in vitro*? This is dependent on the nature of the electron donor *in vivo* and more detailed study of this *in vivo* reaction with physiological electron donors in a range of bacterial species is required.
- *In vivo* is N₂OR actually damaged within a cell by prolonged exposure to oxygen and if so is this damage repairable or is *de novo* synthesis required for renewed N₂O reduction?
- To what degree is N₂OR expression, assembly and activity *in vivo* at agricultural sites dependent on biological copper availability?
- What are the functions of 'accessory' gene products that are highly conserved in nos gene clusters? Do they have roles in the assembly of the copper cofactors of N₂OR and/or in expression of the nosZ gene?
- Can we use technical advances in geochemistry and environmental biochemistry to monitor a wide set of parameters, both of the soil and the bacterial processes, in field studies to allow identification and ranking of the various factors that regulate N₂O production and consumption?
- Can models be developed that are robust enough to feed in time-series of fluxes and expression data from laboratory-based studies into the field?

the enzymology of denitrification could help to solve the N₂O emission problem, at least in part. We suggest that management options will best be informed by an approach that models N₂O emissions from denitrifying bacteria in response to environmental parameters and that is underpinned by robust kinetic measurements derived from ongoing studies of the enzymes of denitrification and, in particular, N₂OR. This will provide vital currently missing kinetic information in existing models on soil N₂O production, such as DNDC [53] or DAYCENT [54]. However, central to the success of such an approach is the hypothesis that the key drivers of N₂O production and consumption at the cellular level are the same and have the same ranked significance, regardless of the physical scale being considered – for example soil aggregate, plot, field or region [55]. Technical limitations currently hinder such knowledge, but only if consistency across scales is indeed true can responses that are measured on the microscale be simply mathematically extrapolated to predict accurately responses at the landscape scale. The teasing out of these possibilities requires a combination of reductionist and holistic approaches, with considerably more mechanistic work required at the molecular level *in vitro* and in further *in vivo* studies, as well as modelling of field conditions for optimal cell growth to obtain completion of the denitrification pathway. Field studies must monitor a wider set of parameters, both of the soil and the bacterial processes taking place, to allow identification and ranking of various factors. Such studies need to take advantage of technical advances facilitating direct measurement of N₂ production against a high atmospheric N₂ concentration if regulation of N₂OR is to be measured *in situ*. Only when we have greater understanding of the production and reduction of N₂O (Box 3) will it be possible to provide farmers with more precise prescriptions to minimize N₂O emissions for, say, application of nitrogenous or copper fertilizer, SOM management and, where necessary, liming of crops or grasslands with specific characterized carbon and nitrogen traits.

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