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]. 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 1900s, 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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N2O emission, which the Kyoto Protocol's successor will even have the gene for N2OR. Likewise, denitrifying fungi NO2 (nitrate reduction regulatory protein), which sense NO, and nitric oxide reduction regulatory protein) and NarR that the enzyme nitrous oxide reductase (N2OR) or the from soils carrying out bacterial denitrification implies that the enzyme nitrous oxide reductase (N2OR) or the contribution that ammonifying bacteria make to global N2O emissions is not known but should be addressed. In fungi, the enzymes that reduce NO3 to ammonium (NH4+) (so-called ammonifiers, see Box 1) can also produce N2O as a by-product, but most do not have an enzyme that can consume it. The contribution that ammonifying bacteria make to global N2O emissions is not known but should be addressed. In fungi, the enzymes that reduce NO3 and NO2 have similarities to those of denitrifying bacteria [15], but the enzyme that detoxifies NO to produce N2O is a cytoplasmic enzyme [28]. Hence it is a potential producer, not a consumer, of N2O. Although not a major focus of this article, bacteria that reduce NO3 to ammonium (NH4+) (so-called ammonifiers, see Box 1) can also produce N2O as a by-product, but most do not have an enzyme that can consume it. The contribution that ammonifying bacteria make to global N2O emissions is not known but should be addressed. In fungi, the enzymes that reduce NO3 and NO2 have similarities to those of denitrifying bacteria [15], but the enzyme that detoxifies NO to produce N2O is a cytoplasmic enzyme [28]. However, fungi also are thought not to have an N2OR and as such are also net emitters of the greenhouse gas.

There are several enzymatic and microbial routes to N2O production. However, the bacterial N2OR, is the only known enzyme capable of reducing N2O to N2. The reaction, shown in Equation 1, is highly favoured thermody-
N₂O is a powerful oxidant with respect to N₂, as shown by the high positive redox potential at pH 7, E° = +1.35 V vs NHE. However, N₂O binds very poorly to metal ions and is difficult to activate towards its reduction. Biology has overcome this problem by evolving in N₂OR a unique enzymatic machinery — (indicated by the large negative value of the free energy, ΔG°).

\[
\text{N}_2\text{O} + 2 \text{e}^- + 2 \text{H}^+ \rightarrow \text{N}_2 + \text{H}_2\text{O} \\
(\Delta G° = -340 \text{ kJ mol}^{-1} \text{ and E}° = +1.35 \text{ V}) \quad \text{[Equation 1]}
\]

**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 N₂O 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 (Fe₉₃) site magnetically coupled to form a dinuclear centre [88]. NorB shares the same overall architecture with heme copper oxidasases [89,90], and homology modelling shows that the heme:Fe₉₃ dinuclear centre, at which N₂O is formed, is buried in the lipid bilayer. The modelling also envisons 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 protonmotive 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 N₂O 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 protonmotive 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 N₂O reduction makes very little difference to the bioenergetics of the bacterium. This is perhaps reflected by several denitrifying bacteria that can reduce nitrate to N₂O but do not have a gene encoding for a N₂O reductase, so that denitrification terminates in N₂O release.

**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 (Δψ) 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 bc₅, cytochrome bc₅; Cyt c₅₅₀, cytochrome c₅₅₀. As the diagram flows from left to right, the electron acceptor becomes progressively more reduced with NO₃⁻ being reduced to N₂ gas.
interface, implying that the dimer is the catalytically active unit (adapted from Refs [41x187]plasm. The electron donors to 
P. denitrificans
multi-copper-sulfide centre, so-called Cu Z, that is capable making seven in total. \([\text{Cu}_4\text{S}]^{n+}\) can undergo a two-electron redox cycle from the homodimer brings the Cu A centre of one monomer within them to proceed at a useful rate. The head-to-tail alignment of the protomers in the CuZ site in the other monomer. Thus electron transfer must take place across 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 \(\beta\)-barrel and a seven-bladed \(\beta\)-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 CUA to CUB. The CUA and CUB centres are \(-40\) \(\text{Å}\) 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 CUA centre of one monomer within \(-10\)–\(-12\) \(\text{Å}\) of the catalytic CUB 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 CUA, showing the binuclear thiol-bridged copper centre that binds to the protein via two histidine ligands and undergoes a one-electron redox change, between \([\text{Cul-CuII}]\) and \([\text{Cul-CuI}]\). (d) Molecular structure of the catalytic centre, CUB \([15–17,19,33,56–60]\), a copper-sulfur cluster \([\text{Cu}_2\text{S}]^{n+}\). Each copper ion is ligated by one or two histidine residues, making seven in total. \([\text{Cu}_2\text{S}]^{n+}\) can undergo a two-electron redox cycle from the mixed-valence state \([\text{CuI(CuII)}\text{S}]\) to the all-copper(I) state \([\text{Cu}_{2}\text{S}]\) [61,62]. The linear molecule N2O is thought to bind along one Cu–Cu edge of the cluster as shown [63].

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 \(\beta\)-barrel and a seven-bladed \(\beta\)-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 CUA to CUB. The CUA and CUB centres are \(-40\) \(\text{Å}\) 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 CUA centre of one monomer within \(-10\)–\(-12\) \(\text{Å}\) of the catalytic CUB 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 CUA, showing the binuclear thiol-bridged copper centre that binds to the protein via two histidine ligands and undergoes a one-electron redox change, between \([\text{Cul-CuII}]\) and \([\text{Cul-CuI}]\). (d) Molecular structure of the catalytic centre, CUB \([15–17,19,33,56–60]\), a copper-sulfur cluster \([\text{Cu}_2\text{S}]^{n+}\). Each copper ion is ligated by one or two histidine residues, making seven in total. \([\text{Cu}_2\text{S}]^{n+}\) can undergo a two-electron redox cycle from the mixed-valence state \([\text{CuI(CuII)}\text{S}]\) to the all-copper(I) state \([\text{Cu}_{2}\text{S}]\) [61,62]. The linear molecule N2O is thought to bind along one Cu–Cu edge of the cluster as shown [63].

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 NO3\(^{-}\) 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

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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 N\textsubscript{2}OR for reducing N\textsubscript{2}O to N\textsubscript{2}. 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 N\textsubscript{2}O. The question then arises of whether denitrifying bacteria have the capacity to sense copper and to regulate N\textsubscript{2}OR synthesis in a copper-dependent manner. This has not yet been assessed, but it is intriguing that the structural gene encoding N\textsubscript{2}OR, nos\textsubscript{Z}, is co-transcribed with nos\textsubscript{R}, 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 nos\textsubscript{DFY}, 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 nos\textsubscript{Z} [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 N\textsubscript{2}OR. 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 N\textsubscript{2}O emissions.

**Modelling the production and consumption of a potent greenhouse gas**

Simple sequential models for denitrification can be formulated that involve only the K\textsubscript{M} and V\textsubscript{max} of each enzyme for its nitrogenous substrate. In such models, there is no significant accumulation of an intermediate if the K\textsubscript{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 O\textsubscript{2} 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 (UQ\textsubscript{H}\textsubscript{2}) 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 N\textsubscript{2}O at pH 8.5, an observation that is consistent with a simple sequential model. By contrast, at pH 5.5, both NO\textsubscript{2}\textsuperscript{-}/CO\textsubscript{3} and N\textsubscript{2}O accumulated transiently up to 300 \textmu M. A kinetic model to describe the experimental data includes the use of two-substrate kinetics with an estimate of V\textsubscript{max} and two K\textsubscript{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 N\textsubscript{2}OR by O\textsubscript{2}, free nitrous acid or NO. In the latter case, the kinetics of enzyme synthesis and repair require measure-
mitigation 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 N₂O flux.

Mitigating N₂O emissions by enhancing the transformation of N₂O to N₂

Globally, we stand on the brink of some major opportunities in agriculture and food production for lowering the production of greenhouse gases, such as N₂O. 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 N₂O is the largest greenhouse gas source in these systems [47]. Surprisingly little is known about this regulation, particularly of N₂O reduction by N₂OR, either at the level of the gene or the protein, yet this enzyme should surely be central to any strategy for the mitigation of N₂O emission. Direct measurements of N₂O reduction in the field are scarce, in part due to difficulties of quantifying N₂ production against a large background atmospheric concentration. Most existing knowledge is inferred from experiments in controlled environments utilizing stable isotope approaches or N₂-free headspaces, or from field experiments involving the application of 10 kPa acetylene as an inhibitor of N₂OR. 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 N₂O production and reduction in the field are thought to be O₂ partial pressure, nitrogen and carbon availability, and soil pH [24]. The N₂O mole fraction, that is, the molar ratio between N₂O and (N₂O+N₂), represents the relative proportion of nitrogen lost as N₂ and N₂O 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 O₂ concentration [48]. It can also be high after addition of NO₃⁻ fertilizers because at concentrations of >10 mg NO₃⁻ g⁻¹ soil NO₃⁻ is preferred over N₂O as an electron acceptor [21].

The ability to denitrify is phylogenetically diverse, and recent developments in techniques for quantifying N₂O 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-N₂O emission from agriculture. Here, we propose that a more reliable approach to mitigating N₂O 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 N₂O to N₂ is not constrained but, instead,
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 monoxygenase (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 CuA and CuZ. 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 CuZ 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
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 N2O 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?

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