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Running title: nitrous oxide production and consumption

Title: Nitrous oxide flux: what microbial physiology can do to mitigate climate change gas production

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Contents

Abstract

Nitrous oxide is a major contributor towards greenhouse gas emissions from agriculture and is the most significant single cause of ozone depletion in the 21st Century. In this chapter, the microbial processes associated with the production and consumption of nitrous oxide are reviewed, with a focus on the role of NosZ in nitrous oxide removal. Recent developments have led to a recognition that two distinct clades of *nosZ* exist, and that diversity exists within and between the clades resulting in functional diversity of NosZ in the organisms that carry them. We point out areas where there are knowledge gaps, particularly a lack of exploration of the comparative biochemistry of NosZ from organisms beyond a few laboratory model species. We discuss the importance of considering how nitrous oxide is measured, and the ways in which factors such as evolutionary selection pressure, regulation, and biochemical organisation impact on the eventual activity of nitrous oxide reduction in biological ecological systems. This is followed by a set of perspectives on how we might apply our current and future knowledge to mitigate atmospheric nitrous oxide accumulation for global benefit.

Abbreviations

ANAMMOX	Anaerobic ammonia oxidation
AOA	Ammonia-oxidising archaea
AOB	Ammonia-oxidising bacteria
COMAMMOX	Complete oxidation of ammonia
CRDS	Cavity ring-down spectroscopy
EC	Eddy Covariance
ECD	Electron capture device
FT-IR	Fourier Transform – infra red
GC	Gas chromatography
GHG.	Greenhouse Gas
IPCC.	Intergovernmental Panel on Climate Change
IRMS	Isotope ratio mass spectrometry
NAP	Periplasmic nitrate reductase
NAR	Membrane-bound nitrate reductase
NCD	Nitrifier-coupled nitrification
ND	Nitrifier denitrification
NN	Nitrifier nitrification
NOR	Nitric oxide reductase
NOS	Nitrous oxide reductase
NOX	Nitrite oxidation
OA-ICOS	Off-axis integrated cavity output spectroscopy
pmf	Proton motive force
QCL	Quantum cascade laser
UNEP.	United Nations Environment Programme

1. Introduction

1.1. Environmental significance of nitrous oxide

By 2050 the human population is expected to reach 9.7 billion (United_Nations, 2022). Sustaining an ever-increasing global population requires continued improvements in agricultural yield, which will involve further technological developments. Nitrogen availability is a major determinant limiting crop growth, and indeed the availability of nitrogen-rich fertilizer synthesised via the Haber-Bosch process has underpinned the improved yields in agriculture that has enabled increasing agricultural production over the last 100 years. Whilst this process has been crucial to supporting a growing human population (half of the nitrogen in food globally is estimated to be derived from fixation via the Haber-Bosch process), the Haber-Bosch process is responsible for elevated production of the Greenhouse Gas nitrous oxide (N_2O is responsible for 6% of global warming (IPCC, 2018), with agriculture contributing c. 75% of total global N_2O emissions (C. Wang, Amon, Schulz, & Mehdi, 2021)). N_2O is particularly notable due to its long atmospheric lifetime (> 100 years (Prather et al., 2015)), and high radiative forcing potential (300 times greater than CO_2, mole for mole) making it a significant long-lasting global warming gas. N_2O is also the most significant cause of stratospheric ozone depletion in the 21st Century (Ravishankara, Daniel, & Portmann, 2009).

The United Nations Environment Programme (UNEP) Global Nitrous Oxide Assessment (United_Nations_environment_programme, 2024) set out the state of the problem, and the required solution, as of 2024. N_2O emissions have increased by 40% since 1980, and, without abatement, are projected to increase by a further 30% by 2050. The UNEP assessment proposes a 40% reduction in anthropogenic N_2O emissions by 2050, which would avoid the equivalent of 6 years' worth of current CO_2 emissions, plus the benefits of reversing ozone depletion and improved air quality that would prevent 20 million premature deaths.

It is worth reflecting for a moment on Haber's invention of a chemical means to generate fixed nitrogen, and the unforeseen impacts. Invented in response to dwindling supplies of nitrate for munitions in the early 20th Century, and hence enabling industrialised killing on previously unknown scales, agricultural gains as a direct consequence of Haber's process support half the world's population's food supply. Haber himself became deeply concerned about the potential long term consequences of his invention, predicting it would cause an overwhelming environmental imbalance leading to excessive plant growth, strangling human civilisation (Labatut, 2020). Obviously this outcome did not unfold as predicted; whilst the nitrogen cycle has become functionally unbalanced, the resultant major environmental catastrophic consequence has instead been the accelerated production of atmospheric nitrous oxide, heating the planet and depleting its protective ozone layer.

1.2. Nitrous oxide: chemistry and biology

In 1799, a twenty year old laboratory assistant (who would later become leading Chemist Sir Humphry Davy) decided it would be a good idea to expose himself to the effects of nitrous oxide, generated *in situ* by heating ammonium nitrate (West, 2014), in the sort of exercise that would cause current research Ethics Committees to feel even more faint than the subject of this experiment. The euphoria the gas produced led to its well-known common name "laughing gas". N_2O became a valuable anaesthetic in medicine, and on the other hand its recreational misuse has led to its classification as a class C drug in the UK since 2023 (Rough, 2023). Nitrous oxide was originally made by thermal treatment of ammonium nitrate - the self-same chemicals have become the source of nitrous oxide production through their use in fertilizer formulations throughout world agriculture.

Nitrous oxide (NCBI, 2025) is a linear molecule with the form $N^-{=}N^+{=}O$. It is a colourless, faintly sweet-tasting gas at room temperature (its boiling point is -89 °C). Chemically, nitrous oxide can be generated by heating ammonium nitrate at 250 °C, the resulting gas being quite

inert at room temperature or dissolved in aqueous solution. N_2O is *thermodynamically* unstable, and can be dissociated by fission of the weaker N-O bond in the reaction $N_2O \rightarrow N_2 + ½ O_2$. However, the activation energy for this reaction is high (~250 $kJ.mol^{-1}$) and so the reaction only occurs spontaneously at high temperatures (> 600 °C).

Nitrous oxide is an intermediate in the biological nitrogen cycle (Figure 1), being an essential intermediate in the process of denitrification (the step-wise reduction of nitrate to dinitrogen gas, which is a typically anaerobic respiratory process in bacteria) and also a side product of nitrification (the oxidation of ammonia to nitrite and nitrate). Nitrous oxide thus produced can be subsequently removed biologically via nitrous oxide reductase, a specific and apparently unique enzyme, capable of this function under physiological conditions. [Insert Figure 1 here]

1.2.1. Nitrous oxide production and reduction in denitrification

Denitrification is an alternative respiratory pathway, predominantly used by bacteria when oxygen is unavailable (Berks, Ferguson, Moir, & Richardson, 1995). Whilst often treated as a pathway, the individual steps are more-or-less independent, and are not always shared within the same cell, species or even environmental setting. The reduction of nitrate to nitrogen is thermodynamically favourable, and, like the reduction of oxygen to water, can be used to conserve energy in the form of ATP generation via a respiratory chain that leads to generation of a proton motive force (pmf).

Nitrate is reduced to nitrite in a two-electron reduction. Multiple different nitrate reductase enzymes are able to carry out this process, but in all the known cases, the enzymes are characterised by the possession of a molybdenum-based cofactor at which the catalysis of nitrate reduction occurs (Gonzalez, Correia, Moura, Brondino, & Moura, 2006). Respiratory systems in which nitrate reduction is associated with membranes lead to the generation of a pmf as electrons flow from NADH to nitrate, a greater pmf being generated when scalar protons

are consumed in the cytoplasm via the membrane-bound nitrate reductase (NAR), that has its catalytic site facing the cytoplasm, than when nitrate is reduced outside the membrane via the periplasmic nitrate reductase (NAP).

Nitrite is reduced to nitric oxide (the free radical $NO^{\cdot}$) via nitrite reductase. Two distinct nitrite reductases have different catalytic mechanisms: NirK, which has a copper as the catalytic redox centre (Dodd, Hasnain, Abraham, Eady, & Smith, 1997), and NirS which has a d_1-haem (in fact an isobacteriochlorin) at the active site (Williams et al., 1997). Each of these enzyme types catalyses a one-electron reduction of nitrite to the nitric oxide product, and are linked to respiratory chains for their source of electrons, which are in turn coupled to pmf generation. These denitrifying nitrite reductases are not to be confused with other nitrite reductases that produce ammonia as a product in a six electron reduction of nitrite (Einsle, 2011). The production of NO is the committal step of denitrification, as it produces the first gaseous intermediate. Abundance and diversity of *nirS* and *nirK* are frequently used to monitor denitrification in environments, given their centrality to this committal step. Unlike the other steps of identification, this step is usually tightly coupled to the onward reduction of NO to N_2O, in order to limit the accumulation of toxic NO.

Nitric oxide is reduced to nitrous oxide by nitric oxide reductases. Canonical nitric oxide reduction (NOR) is carried out by integral membrane-bound enzymes evolutionarily related to the haem-copper oxidases that reduce oxygen to water in aerobic respiration (Abraha, Gelfand, Hamilton, Chen, & Robertson, 2018). This class of nitric oxide reductase splits into at least two subcategories: qNOR -in which the electron-carrying subunits associated with the haem-copper oxidase-like (noting that the active site copper is replaced by non-haem iron in NOR) core subunit receive electrons from the quinol pool, and cNOR -in which the electron-carrying subunits receive electrons from reduced cytochromes c. The latter will typically be associated with greater conservation of energy in the form of pmf generation, due to the proton

translocating activity that takes place in the cytochrome bc_1 complex (which translocates protons across the membrane coupling this to the oxidation of quinols by cytochromes *c*). These nitric oxide reductases catalyse the two-electron reduction of two NO molecules to form one N_2O (and one molecular of water). This reaction is thermodynamically highly favourable (with a standard reduction potential of +1175 mV, which is more favourable than reduction of oxygen to water: 820 mV), and occurs fast enough in relevant biological systems to keep NO at low concentrations at steady state. Other nitric oxide-reducing enzyme systems in bacteria include flavorubredoxin (Gardner, Helmick, & Gardner, 2002) in non-denitrifying, but facultative anaerobic enterobacteria. The instability of NO and its willingness to bind to metal centres makes it easy to understand that NO reductase activity can have evolved multiple times.

As noted above, nitrous oxide is thermodynamically unstable but kinetically stable at physiological temperatures in the absence of appropriate catalysis. In denitrification, the nitrous oxide reductase (NOS) catalyses the two-electron reduction of N_2O to N_2 plus water (Hein & Simon, 2019). Like the other denitrifying reductases, this is linked to electron flow through respiratory chains that are coupled to the generation of a pmf. It appears that there is a single type of enzyme that catalyses the reduction of N_2O to N_2. The enzyme is characterised by a special copper cofactor (mu-4-sulfido-tetra-nuclear copper ion) that appears unique to the nitrous oxide reductase (Chen, Gorelsky, Ghosh, & Solomon, 2004). This site is able to ligate N_2O, bring about its two-electron reduction to N_2 and then release the product. It is worth noting at this point that, from the bacterial point of view, the reduction of N_2O does not need to be tightly coordinated with the reduction of nitric oxide to nitrous oxide. N_2O is rather inert, has limited influence on other microbiological processes, and so can be allowed to accumulate to high concentrations in the environment in which it is generated. On the other hand, nitrous oxide reduction is thermodynamically advantageous given the very high redox potential of nitrous oxide reduction (+1355 mV), and so its removal can be advantageous for denitrifiers or even specialist nitrous oxide-reducing bacteria that may not possess the capacity to carry out

the other steps of denitrification. Nitrous oxide reduction is thermodynamically favourable, compared even to oxygen reduction, and indeed Stuart Ferguson showed that N_2O reduction can continue in the presence of oxygen (Bell & Ferguson, 1991). That said, rates of nitrous oxide depend on the relevant genes being activated, the concentration of nitrous oxide being sufficient (relative to the binding affinity for the substrate that the enzyme can achieve), and the enzyme retaining activity (NosZ is highly sensitive to oxygen at least in vitro). These issues will be discussed over the course of this work.

1.2.2. Nitrous oxide production in nitrification

Autotrophic/lithotrophic nitrification is the process by which bacteria and archaea use mineral nitrogen sources as an energy source to support growth. Typically this lithotrophic lifestyle will be accompanied by autotrophic carbon dioxide fixation. Ammonia-oxidising bacteria (AOB) and archaea (AOA) oxidise ammonia to nitrite (Prosser & Nicol, 2012), using the thermodynamic favourability of this oxygen-dependent oxidation to drive generation of a pmf and hence ATP production. The process also generates enough low potential reductant to produce NADH, and thus drive CO_2 fixation. The microbes that carry out this process are abundant, especially in environments which are carbon-poor. Nitrite oxidisers (NOX) use nitrite as an electron donor, producing nitrate as a product (Starkenburg et al., 2006), and using this to drive pmf and reductant generation. Some microbes have now been identified that can oxidise ammonia fully to nitrate (COMAMMOX) (Daims, Lucker, & Wagner, 2016). In AOA/AOB, ammonia is oxidised via molecular-oxygen-dependent ammonia monooxygenase which generates hydroxylamine. Subsequently the hydroxylamine oxidoreductase (HAO) oxidises hydroxylamine (NH_2OH) to nitrite, with nitric oxide proposed to be an obligate intermediate (Caranto & Lancaster, 2017), including in HAO-containing methanotrophs (Versantvoort et al., 2020). The step required for oxidation of nitric oxide to nitrite may be NirK, or could be a spontaneous reaction with oxygen,

but this is yet to be unambiguously determined. The status of NO as a free intermediate on the route to nitrite remains controversial and is not supported by a more recent study (Choi, Chaudhry, & Martens-Habbena, 2023), in which the presence of an NO scavenger has only a very limited effect on nitrite production under oxygen replete conditions, but completely stymies nitrous oxide production (nitrification-denitrification) under oxygen limitation. This suggests NO may not be a freely diffusible intermediate of nitrification to nitrite, but it is a required intermediate in the pathway to N_2O.

The production of N_2O via nitrification occurs via two main mechanisms: N_2O released as a product of the hydroxylamine oxidase as a side-product (nitrifier nitrification, NN), and via the denitrification of product nitrite to nitrous oxide. This latter process may occur as catalysed reactions within the nitrifier itself (this is referred to as nitrifier denitrification, ND) or reactions catalysed by separate heterotrophic denitrifiers occupying the same community (nitrifier-coupled denitrification, NCD). It has been possible to distinguish between these mechanisms, for example by using isotopically labelled ammonium, nitrate or nitrite. An early study using this approach indicated that in the model species nitrifier *Nitrosomonas europaea*, that nitrous oxide is derived from nitrite via denitrification in this species (Poth & Focht, 1985). Further methodological developments have used the distribution of isotopomers of N_2O (see Measurement section 3.3.2.) to indicate that nitrous oxide derived from leaky activity of hydroxylamine oxidase gives a different signature to nitrous oxide derived from nitrite (Sutka, Ostrom, Ostrom, Gandhi, & Breznak, 2003). In complex communities such as wastewater nitrifying sludge, it appears that both NN and ND are important processes, but with ND dominant except at high oxygen concentrations (e.g. (L. Peng, Ni, Ye, & Yuan, 2015)). The majority of nitrous oxide emissions from streams globally is reckoned to be derived from nitrifier denitrification (S. Wang et al., 2024). There is a distinction in terms of N_2O production by AOB and AOA. The ammonia-oxidising archaea are generally lacking nitrite and nitric oxide reductases, and produce lower fluxes of nitrous oxide than AOB (Hink, Nicol, & Prosser, 2017).

Stieglmeier and colleagues put forward that N_2O in AOA is generated through N-nitrosation not nitrifier denitrification (Stieglmeier et al., 2014). AOA activity has been reported to be stimulated under low oxygen conditions, and that this influences N_2O production (Qin et al., 2017). The complete COMAMMOX nitrifiers produce only low levels of N_2O (Kits et al., 2019), like the AOA, presumably because the nitrite is actively oxidised to nitrate, limiting nitrifier denitrification of nitrite.

1.2.3. Other modes of nitrous oxide production

Whilst the majority of attention has been focused on bacteria, fungi have been reported to carry out denitrification, and indeed the enzyme responsible for producing nitrous oxide from nitric oxide in *Fusarium oxysporum* was reported in 1993 (Nakahara, Tanimoto, Hatano, Usuda, & Shoun, 1993). Some fungi can also express nitrate and nitrite reductases (Kobayashi et al., 1996), and thus this is potentially a major route for N_2O production. Indeed there is a significant body of work claiming that fungal denitrification dominates denitrification in some environments (see *e.g.* (Long, Heitman, Tobias, Philips, & Song, 2013) (Huang et al., 2023; Xiong et al., 2024)). It has been noted however, that some field studies into fungal denitrification are reliant on the use of biocides to distinguish fungal from bacterial activity, and so should be treated with a certain amount of caution (Butterbach-Bahl, Baggs, Dannenmann, Kiese, & Zechmeister-Boltenstern, 2013).

Other routes to nitrous oxide include direct chemical routes for the production of nitrous oxide from other nitrogen containing intermediates such as nitrite and hydroxylamine (chemodenitrification, see e.g. (Hunt et al., 2024; Yoon, Song, Phillips, Chang, & Song, 2019)). In addition to autotrophic / lithotrophic nitrifiers, some heterotrophs are reported to be capable of nitrification. This has been under-explored in the literature, but there has been some recent interest in re-exploring this topic (Jin, Chen, Yao, Zheng, & Du, 2019; Lenferink, Bakken, Jetten, van Kessel, & Lucker, 2024; Lu et al., 2024).

1.3. A manifesto for action

From a microbial physiologist's perspective, how can we influence nitrous oxide emissions? The UNEP Nitrous oxide assessment 2024 sets out aims for a major reduction in nitrous oxide in the coming years. The processes are grounded in microbial function and we must pursue this action agenda with purpose. This article aims to explore ways in which we might address this, through understanding to inform monitoring & assessment of nitrous oxide sources and sinks, the use of ecological and agricultural management strategies for mitigation and the development and implementation of new biotechnological solutions.

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2. The distribution, means and activity of nitrous oxide removal

The known mechanism for the degradation of nitrous oxide is via the N_2O reductase NosZ. This enzyme is characterised by the possession of a specialised multi-copper centre with a unique cofactor structure (Chen et al., 2004). *nosZ* is contained in an operon along with nosL, whose gene product is a dedicated copper chaperone essential for correct NosZ cofactor assembly (Bennett et al., 2019). Assembly of the active site Cu_Z site also requires the ABC transporter NosFY and periplasmic accessory protein NosD (Zhang, Wust, Prasser, Muller, & Einsle, 2019), all of these genes being standard components of diverse *nos* operons. Additional components of *nos* operons are more variable, and include electron transport proteins and regulators that are divergent amongst *nos*-carrying organisms.

The story of nitrogen cycle microbial physiology is one of dogmas, which have been repeatedly swept away by discovery. Respiratory nitrate reduction was carried out by the membrane-bound nitrate reductase until the periplasmic nitrate reductase was discovered (Bell, Richardson, & Ferguson, 1990). Denitrifying bacteria strictly contained only NirS or NirK type nitrite reductase until both were found and shown to function in the same strain (Sanchez & Minamisawa, 2018). Nitric oxide reductase used cytochrome *c* as an electron donor until qNOR was discovered (Cramm, Siddiqui, & Friedrich, 1997). Nitrification was a monophyletic bacterial process until the ammonia-oxidising archaea were discovered (Konneke et al., 2005). Nitrifiers either oxidised ammonia or nitrite until the COMAMMOX was found (Daims et al., 2015). Denitrification was the only means of removing fixed nitrogen from the biosphere until ANAMMOX was discovered (Strous et al., 1999; van de Graaf et al., 1995). Nitrous oxide reductase remains the preserve of a single enzyme type, albeit that the canonical NosZ (clade I) has been joined by a second group of NosZ types (clade II) (Jones, Graf, Bru, Philippot, & Hallin, 2013; Sanford et al., 2012). Perhaps this will remain the case, although history indicates that there is more functionally redundant diversity hiding in nature. It is possible that the specialist chemistry required to drive the kinetically challenging activity, and the limited bioenergetic gain that is achieved by possessing this activity in many ecological settings, has limited the selection pressure for nitrous oxide reductase evolution leading to only a single biochemical mechanism for nitrous oxide removal. That said, nitrous oxide removal can be a significantly valuable driver of microbial selection in some settings (Kim et al., 2022). It is worth noting that iron-based chemical catalysts for N_2O degradation have been developed (Aranifard, Bell, Keil, & Heyden, 2021), so there is the possibility that a different bioinorganic catalytic mechanisms for N_2O reduction could be envisaged.

2.1. Nos I and II and their distributions, abundances and function

A literature noting different clades of *nosZ* arose first around 2012, increasing exponentially until 2018, and since then stable (the highest number of publications being 18 in 2024), with 92 papers altogether to date, according to pubmed. From the initial discovery of two separate NosZ clades, it was found that "typical" NosZ (clade I) is most commonly associated with denitrifying organisms that possess an entire denitrification pathway including nitrate reductase, nitrite reductase and nitric oxide reductase, whereas the atypical (clade II) NosZ is found in organisms lacking other denitrification reductases (Sanford et al., 2012).

PCR-based methods have been developed and optimised to recover *nosZ* genes from the two clades from a variety of environmental settings (Chee-Sanford, Connor, Krichels, Yang, & Sanford, 2020; Keeley et al., 2020; Kim et al., 2020). This methodological approach is now being enhanced by metagenomic sequencing and assembly-based methods (Schacksen & Nielsen, 2024).

The initial observation that clade I is identifiable with denitrification and clade II is associated with non-denitrifiers has been borne out in subsequent studies, although the distinction is not absolute. A recent analysis of the distribution of *nosZ* genes of the two clades is explored by Intrator and colleagues (Intrator, Jayakumar, & Ward, 2024). The significance of the clade II *nosZ* grouping is evident. For example, Bertagnolli and colleagues (Bertagnolli, Konstantinidis, & Stewart, 2020) used collections of metagenomic data from marine environments to show that (i) clade II *nosZ* is dominant, and (ii) that the clade II *nosZ* sequences are typically associated with non-denitrifiers. That said, the denitrifying bacterium *Thauera linaloolentis* possesses both a clade I and a clade II *nosZ* gene (Semedo, Wittorf, Hallin, & Song, 2020). Interestingly, the clade I is upregulated in the presence of nitrate, but the clade II enzyme is upregulated in the absence of nitrate, in line with the idea that clade II NosZ is associated with non-denitrifying conditions.

Several studies indicate an ecological distinction between the roles of *nosZ* from the two clades. In agricultural soils, clade II *nosZ*-containing partial or non-denitrifiers are favoured under oxic incubation, in contrast to clade I *nosZ*-containing strains, dominantly denitrifiers and favoured under a regime of long anoxic spells (Sennett et al., 2024). Jiang (Jiang, Liu, Wang, Sun, & Zhu, 2024) found a switch in *nosZ* clade type in rice paddies, clade II dominating during tillage, clade I in fallow periods. Clade II *nosZ* seems particularly important for nitrous oxide removal under acidic conditions (Sun et al., 2024), in lakes (Song et al., 2024), aquifers (Hunt et al., 2024), and soil systems under grazing (F. Zhang et al., 2024). Graf (Graf, Jones, Zhao, & Hallin, 2022) found that clade I *nosZ* is more abundant in the rhizosphere than bulk soil and vice versa for clade II, the same study showing differences also in phylogeny-based community composition between these compartments.

Not all of the work on this topic supports the view for an ecological distinction between NosZ clades, Lin et al (Lin, Hu, Deng, Yang, & Ye, 2023) found that whilst clade I and clade II *nosZ* were housed in phylogenetically distinct chassis organisms (the former dominant in Alpha and Beta-proteobacteria, the latter in Gemmatimonadetes, Verrucamicrobia, Gamma-proteobacteria and Chloroflexi) they were similarly distributed in tropical wetlands, indicating that the clades share a similar ecological function there.

Compared to the other reactions of denitrification, nitrous oxide reduction is sensitive to low pH (Cuhel et al., 2010). Recent work by Sun (Sun et al., 2024) has found *nosZ* (clade II) in acidic tropical forest soils, and that this distribution is common in a range of soil microbiomes from pH 3.5 to 5.7, indicating a broad distribution of N_2O reduction potential (if not necessarily activity) in such settings.

As well as sensitivity to pH, nitrous oxide reduction is also particularly sensitive to oxygen compared to the other denitrification reductases (both in terms of expression and activity). Analysis of clade I and clade II NosZ by Wang et al recently (Z. Wang, Vishwanathan,

Kowaliczko, & Ishii, 2023) indicated that (i) there is no distinction between the two clades in terms of oxygen sensitivity in intact cells reducing nitrous oxide, (ii) the lack of association between oxygen tolerance, sensitivity and intolerance indicates that the oxygen response operates at a cellular level (through for example oxygen removal mechanisms) rather than at the level of the enzyme *per se*. Wang's data shows NosZ of *Pseudomonas (Stutzerimonas) stutzeri* to be completely oxygen tolerant, whereas similar experimental comparative analysis (Zhou et al., 2021) showed NosZ from the same organism to be fully inhibited in the presence of oxygen. Aerobic denitrification studies continue to be inconsistent and difficult to interpret, as they have been for decades.

Clade II *nosZ* is carried by some strict aerobes (eg. *Gemmatimonas aurantiaca*), in which case the N_2O reduction activity appears to be advantageous under brief periods of anoxia - N_2O reduction here allows survival, but not growth (Park, Kim, & Yoon, 2017). The significance of clade II aerobe N_2O reduction as a ecophysiological function is emphasised by (Sennett et al., 2024) who demonstrated the clade II dominance in aerobic non-denitrifiers, whereas clade I denitrifiers reduce N_2O in a regime that involves repeated long anoxic periods.

Given the tendency towards clade II NosZ being associated with non-denitrifiers, what evidence is there that these enzymes / organisms may be particularly well adapted to scavenging low N_2O concentrations, thus making them of particular significance in development of engineered solutions to N_2O capture? In aquatic environments clade II NosZ are associated with more successful mitigation of N_2O production in aquifers (Hunt et al., 2024) and shallow lakes (Song et al., 2024). Several studies have indicated that clade II NosZ do indeed have a higher affinity for N_2O than the clade I types. For example, Yoon (Yoon, Nissen, Park, Sanford, & Loffler, 2016) calculated K_s values for clade II NosZ (*D. aromatica* and *A. dehalogenans* to be substantially lower (0.324 ± 0.078 μM and 1.34 ± 0.35 μM, respectively) compared to clade I NosZ from *Ps. stutzeri* (35.5 ± 9.3 μM) and *S. loihica* (7.07 ± 1.13 μM). This is

backed by Zhou's findings (Zhou et al., 2021) where apparent K_M values (assays of N_2O reduction in intact cells) demonstrated sub micromolar K_M for two clade II bearing *Azospira* strains, and >2.5 μM K_M for *Ps. stutzeri* and *P. denitrificans*. This was also consistent with demonstrations of enrichment of higher N_2O affinities in clade II-carrying biofilm isolates in a gas-permeable membrane reactor (Suenaga et al., 2019), On the other hand, Conthe (Conthe et al., 2018) found that under N_2O-limiting conditions clade I carrying strains had a higher affinity for N_2O when judged by the efficiency measure of μ_{max}/K_s. Wang saw no pattern of distinction between clade I or II NosZ enzymatic properties in their study (Z. Wang et al., 2023), including K_M. What is notably lacking in all of these studies is the dissection of NosZ away from its setting within the host organism, i.e. no protein biochemistry of the clade II NosZ. That said, Wang does present some analysis of alpha-fold based modelling of the NosZ which indicates that all the N_2O reductases produce predicted structures that map closely on to the experimentally-determined structures, but that the Z scores (goodness of match to the experimentally determined structure) are lower for clade II structures than clade I -which is not unexpected given the evolutionary distance.

To explore the structural similarity or otherwise between clade I and II NosZ, we used AlphaFold (version 3) to predict the structure of a clade II NosZ representative (that from *Campylobacter fetus*). A high confidence structure was obtained and this was overlaid on the structure of NosZ from *Paracoccus denitrificans* (clade I) using the align tool in Pymol in order to visualise the two structures (Figure 2) [Insert Figure 2 here]. The structural similarity is remarkable, given the two proteins share only 33% identity, even around the cofactor binding sites (the presence of which were not factored in in the model build). Overall, the deviation between the two structures has an RMSD = 1.134 Å from the 2674 aligned atoms. Figure 2 shows the high degree of similarity in the positions of the conserved His ligands that coordinate the coppers in the active site Cu_Z. Other key catalytic residues, equivalent to K397 and E435 from the *P. denitrificans* NosZ are conserved and their positions in the predicted structure are

near identical. Within the pocket above the Cu_Z site *P. denitrifcans* contains two additional amino acid residues M570 and N189 (these are derived from the other subunit in the NosZ dimer). In *C. fetus* these residues are replaced with two Leucine residues (L609 and L215). Whilst this potentially has an impact on the binding of N_2O, it is not a clade-specific mutational signature. Whilst Met and Asn are at these positions in characterized clade I NosZ (*e.g. P. denitrificans*, *Ps. stutzeri*, *Ps. aeruginosa*, as well as *Sinorhizobium*, *Bradyrhizobium*, *Achromobacter*, *Shewanella* and *Brucella* species), Leucines are present in some, including the predicted NosZ gene products from *Ralstonia pseudosolanacaerum* and *Cupriavidus necator*. Nonetheless, the influence of these factors on access and affinity of N_2O should be of experimental interest.

In addition to the two conserved domains, the betapropeller domain that contains Cu_Z and the cupredoxin-like Cu_A domain *C. fetus* NosZ contains are further two domains, an alpha helical bundle and a fourth domain that appears to house a single *c*-type haem. *C. fetus* NosZ is found in a gene cluster that contains other key accessory genes as well established for other Nos clusters such as the ATP-binding cassette transporter NosDFY and the copper chaperone NosL. Additionally, the cluster in *C. fetus* encodes homologues of the quinol dehydrogenase NapGH (Brondijk, Nilavongse, Filenko, Richardson, & Cole, 2004), and two genes that appear to encode *c*-type cytochromes, presumably also involved in electron transport to nitrous oxide.

3. Predicting nitrous oxide removal

3.1. Modelling nitrous oxide

Effective modelling and prediction of nitrous oxide fluxes in different environments is essential for the generation of nitrous oxide inventories that underpin global models of nitrous oxide emissions, and also as a scientific basis for making management decisions about nitrous oxide

mitigations. Ultimately, it should be possible to explain the rates of nitrous oxide production and removal from rich enough information about the biological composition and organisation in an environment, and the prevailing environmental conditions, given good enough understanding of the underlying structures and controls, and the necessary resolution. Simpler models that account for *enough* to correctly model the nitrous oxide dynamics AND have predictive power is a challenge though. We know, for example, that there is copious variation in nitrous oxide both temporally and spatially, with “hot spots” and “hot moments” of nitrous oxide flux that are hard to explain.

The UNEP Global nitrous oxide assessment (United_Nations_environment_programme, 2024) presents the possibility of a positive future for nitrous oxide control through management strategies, that have been shown in trials to bring benefits in ameliorating nitrous oxide release, particularly from agricultural land, *e.g.* through fertilization formulations, timings and amounts. The Assessment, and many of the literature reviews and meta-analyses focus on outcomes and environmental measures, yet are neutral on the underlying microbiological processes that carry out production and removal of nitrous oxide -assuming these processes will look after themselves. Here we will review some of the recent meta-analyses in this area, before addressing how microbial physiology might advance our understanding and progress.

Li and colleagues (2022) (Z. L. Li et al., 2022) compiled data from >6000 field measurements in over 200 papers, to conclude that the major determinants of global nitrous oxide release from soils are nitrogen (ammonium, nitrate and total) availability, and that a high C:N ratio and a high microbial C:N are correlated with suppressed nitrous oxide emissions. This effect of nitrogen content on nitrous oxide release is also seen in experimental amendment studies. Aronson and Allison, 2012 report short term N_2O increase in response to nitrogen amendment in non-agricultural soils, but this impact is most pronounced in the short term, diminishing over the 23 years of the data collection.

Maaz and colleagues (Maaz et al., 2021) focused on crop yield versus nitrous oxide release in agricultural systems. They conclude that effective management of nitrous oxide release against yield is best achieved through a focus on the metric of N balance, a parameter that is both easy to calculate by farmers and easily understood, speaking as it does of the difference between nitrogen input in fertiliser versus nitrogen content in crops (McLellan et al., 2018).

Other recent meta-analyses have focused on other aspects of the influence of agricultural management on nitrous oxide emissions (Grados et al., 2022), with a focus specifically on how best to manage use of crop residue (shallow incorporation, ensure high C:N ratio, use mature and or digested residue) (Abalos et al., 2022), and on specific crops, e.g. potato in which effects of N, pH, water content are all drivers of nitrous oxide (Ball & Hernandez-Ramirez, 2025).

Whilst these approaches are creating a better understanding of what good land management looks like, there is still plenty of variance in nitrous oxide release that is not explained by environmental variables, opening the way for a more thorough analysis with reference to smaller spatial scale factors such as the microbial behaviour itself in reference to the microenvironments which these microbes inhabit, to the landscape and global scale impact of these factors.

3.2. Microbial traits as drivers of N_2O production and removal

Microbial traits are the underpinning requirements for nitrous oxide production and removal, and understanding the composition, dynamics and diversity of these traits will improve monitoring and manipulation of nitrous oxide production. A combination of environmental measurements and microbial traits should increase our understanding of microbial ecological systems (Krause et al., 2014). Graham et al. (Graham et al., 2016) analysed the impact of microbial community and functional gene analysis on predictability of biogeochemical

processes, and showed, based on 82 studies in different environmental systems, a significant uplift in the predictive power, once molecular microbial analyses were incorporated.

In some studies, microbial functional genes are analysed with a view to explaining changes in nitrous oxide emissions under different regimes, *e.g.* in agricultural settings (Behnke et al., 2022) (Kuusemets et al., 2025), revealing the importance of one or another microbial N-cycling process in leading to N_2O in particular settings regionally, and up to a continental scale (Zhao et al., 2024). The revealing of underpinning changes in the microbial processes under different fertilization or cropping regimes provides a solid basis for future action. Beyond this, Hu (Hu, Chen, & He, 2015) argues that incorporation of N_2O microbial pathways into ecosystem models of N_2O production are essential to improve reliability and drive robust decision-making. This is supported by studies demonstrating the significant impact of microbial functional genes on predicting N_2O emissions from forest soils (Y. R. Peng et al., 2024), rice rotations (P. Xu et al., 2024), and semi-arid grasslands (Y. H. Zhang et al., 2024). Variation in nitrous oxide production seasonally in a waste water treatment plant could be linked to abundance of *nosZ* (Valk et al., 2022). Similarly, the variation in dominance of nitrification or denitrification in different aquatic systems could be understood through changes in relevant gene frequencies (C. L. Wang, Xv, Wu, Li, & Li, 2024). Such thinking and approach is driving calls for the generation of crop-specific N_2O emission factors to be adopted (Shorunke, Helgason, & Farrell, 2025).

3.3. Microbial physiological considerations

To take the microbe's eye view on nitrous oxide turnover, a typical experimental ecological strategy will be to measure the genetic potential for activity in particular settings using amplicon diversity or metagenomics techniques, or to push this one step further and define the transcriptome in a particular complex environmental setting. These studies are powerful and

valuable, adding a biological dimension to our understanding, and have the advantage of being high-throughput enough to reveal differences within and between sites at a variety of spatial and temporal scales. However, the potential activity revealed by the DNA or transcript content does not take enough account of the underlying biological structures and considerations that determine the actual *in vivo* enzyme activity that defines fluxes at a given time, nor the significant impact of those activities on microbial success that drive the microbial community dynamics that define the future, and thus predictability in the ecosystem.

The section that follows will aim to address four key themes: (i) the significance of nitrous oxide to the successful reproduction of microbes (evolution), (ii) the variety of difficulties in correctly determining the properties and activities of microbes in complex environments, and distinguishing between production and consumption (measurement), (iii) the factors that govern nitrous oxide production and consumption at a cellular scale (regulation), (iv) the molecular basis of enzymatic activity, and where the gaps are in our knowledge (biochemistry).

3.3.1. Evolution

How useful is it to maintain and express a nitrous oxide removal pathway? The redox half reaction of nitrous oxide to nitrogen has a high potential -higher than any of the other physiological respiratory reactions in the nitrogen cycle or oxygen reduction, and so from that perspective, nitrous oxide removal should be highly favourable. However, the exquisite sensitivity of N_2O reductase to oxygen means there is considerable risk to investing significantly in N_2O reduction. Unlike the analogous oxygen sensitive process of nitrogen fixation, N_2O limitation is rarely going to be prejudicial to survival and success of a facultative nitrous oxide reducer, and thus special methods to protect nitrous oxide from oxygen sensitivity appear not to have evolved, as they have for N_2 fixers. Nevertheless, N_2O reduction has arisen evolutionarily, and so there are some situations in which a capability to remove nitrous oxide confers advantage, both on denitrifying organisms and those that remove nitrous oxide independently of

the rest of the denitrification pathway. The environmental observations that nitrous oxide production is decreased in environments that have a lower relative nitrogen content (so nitrous oxide is a relatively more useful electron acceptor in respiration), or are wetter (thus more consistently anaerobic, thus nitrous oxide reductase is more stable) is in alignment with these evolutionary imperatives.

Other intermediates in the reductive wing of the nitrogen cycle (nitrite and nitric oxide) are toxic at around their physiological concentrations, and so their removal is dually selectable - for the respiratory benefits of them as respiratory electron acceptors, and for the detoxification. These two imperatives can be seen in the multiple different types of enzymes that exist for their removal and their linkage into cellular reductant availability. Nitric oxide reductases that are tightly coupled to the respiratory chain are seen in model denitrifying bacteria such as *Paracoccus denitrificans* (Carr & Ferguson, 1990), but also a whole range of energetic efficiencies of use are also seen, down to NADPH linked NO reductase processes in the bacterial cytoplasm that serves to remove a toxin, not make ATP (Gardner et al., 2002). Nitrous oxide is mildly toxic, for example through its impact on the stability of vitamin B12-dependent processes (Sullivan, Gates, Appia-Ayme, Rowley, & Richardson, 2013) (e.g. ribonucleotide reductase (Shearer, Hinsley, Van Spanning, & Spiro, 1999), and so this driver should not be blindly dismissed). We have argued previously that the loss of nitrous oxide metabolism is driven in higher N availability systems, in the genus *Neisseria* in particular (Moir, 2011). So, there is fertile ground to explore the dynamics and distribution of *nosZ* types in the biosphere, and their drivers in ecological and experimental systems of study.

3.3.2. Measurement

3.3.2.1. Analytical measurement of N_2O concentrations

In 1957 James Lovelock invented the electron capture detector (ECD) (Lovelock, 1958), which enabled robust and reliable detection of a range of compounds including N_2O. For many

decades since, this technology has been used in conjunction with gas chromatography (GC) to quantify N_2O in discrete gas samples of air, separated into their constituent compounds by the time taken to pass through a column (retention time). If paired with an isotope ratio mass spectrometer (IRMS), the isotopomers of N_2O can be measured this way (see stable isotopes, below). Samples may be collected and stored in gas-tight containers for many months prior to analysis and thus can facilitate investigation of N_2O in remote locations, where samples can be collected in field campaigns and analysed upon return to the laboratory. However, until recently, GC was largely restricted to use in the laboratory, creating an inherent lag between sample collection and data emergence. The discrete nature of GC samples also restricts its application in continuous monitoring of N_2O concentration. And while advances have been made in developing field-deployable GCs (Rapson & Dacres, 2014), the requirement of a power source is a distinct disadvantage. One other limitation of GC with ECD is that whilst it is excellent at measuring N_2O concentrations at ambient and above, it is much less accurate below ambient, which hinders its use in environments where there is a net negative flux (uptake) of N_2O.

Over recent decades, the development of optical approaches to measuring N_2O has seen a step-change in analytical technology. These methods rely on the general principle that certain molecules (in this case N_2O) absorb electromagnetic energy at specific wavelengths (usually within the infrared (IR) spectrum), and so the amount of absorbance is proportional to the concentration of N_2O. Passing a focused light source, such as a laser, through a sample of gas in air and measuring the reduction of the light energy at a detector enables the quantification of the N_2O concentration. The distance travelled by the light energy between source and detector (path length) may be configured as an open path of many metres (~10 – 1000 m), or a closed path, contained within a measurement cell in e.g. a sealed analyser. Since N_2O is present in air at concentrations orders of magnitudes lower than e.g. carbon dioxide (CO_2) and methane (CH_4), the path length required for practical N_2O measurements has been larger than for the other gases, leading to the development of multi-pass cells which rely on

mirrors to reflect the light many times back and forth through the sample in the measurement cell. Sensitivity of the analyser is increased with path length, requiring larger measurement cells, more powerful lasers and thus larger analysers. Several variations of optical approaches have been developed, such as Fourier transformed infrared (FTIR), which measures across the full IR spectrum and can measure several gas species simultaneously, and mid-IR laser spectroscopy where the laser is tuned to a narrower bandwidth and so may measure only one gas. Developments in laser technology, including quantum cascade lasers (QCL), cavity ringdown spectroscopy (CRDS) and off-axis integrated cavity-output spectroscopy (OA-ICOS) have reduced the power required for these analytical techniques; coupled with improvements in mirror design, this has facilitated the development of more compact field-deployable laser analysers. Delivering continuous N_2O concentration data in real-time, this equipment has driven the near-exponential increase in studies since 1995 (Milam-Guerrero, Yang, To, & Myung, 2022). Optical techniques also have the advantage that they are much better than ECD for measuring at low (below ambient) concentrations and can also be calibrated to measure isotopic signatures of N_2O (see stable isotope section below).

A number of other technologies for measurement of N_2O are at various stages of development. These include amperometric techniques (microsensors), which measure the electrical current produced at an electrode when N_2O is reduced (with the current strength proportional to the N_2O concentration), and chemiresistive sensors, which are based on the known change in resistance of metal oxides when exposed to N_2O. These technologies have been restricted by their sensitivity (lower limit of detection) and the temperatures at which they need to operate, but there is considerable scope that a low cost, reliable and accurate sensor for N_2O that provides continuous real-time data is within reach (Milam-Guerrero et al., 2022).

3.3.2.2. Environmental measurement of N_2O fluxes

The most important flux, when considering N_2O, is the total global net flux. This is relatively straight-forward to measure, since N_2O is a well-mixed atmospheric gas with a long lifespan, its atmospheric concentration is relatively homogeneous and therefore periodic concentration measurements allow us to understand how global concentrations change over time. The challenge is to understand N_2O flux at a scale that we can manage. Knowing where and when it is released and what the controlling factors are will enable management practices to be put in place to reduce emissions. Particular consideration should be given to the resolution, temporal and spatial, at which a given technique generates data (Levy et al., 2022) (Figure 3). Here is where N_2O measurement becomes particularly difficult. Environmental fluxes of N_2O are extremely variable in both space and time, characterised by 'hot moments' and 'hotspots' (Groffman et al., 2009). [Insert Figure 3 here]

Hot moments may last several days, and often can be predicted from timing of interventions such as fertiliser application (Keane et al., 2018). In these moments, as much as 20% of a system's annual N_2O emissions may be produced in a few hours (Mummey, Smith, & Bolton, 1997). However, it is not so predictable that fertiliser application will always lead to N_2O hot moments (Keane et al., 2018), even in the same location as previously witnessed. Hot moments are also associated with rain events. Similarly, hot spots are locations which demonstrate extremely localised high fluxes of N_2O, e.g. an area c. 1% of an agricultural field emitting 55% of its total flux (van den Heuvel, Hefting, Tan, Jetten, & Verhoeven, 2009). At the micro scale, <1% of soil volume may be responsible for the total N_2O flux, consisting of the anaerobic centres of soil aggregates (Sexstone, Revsbech, Parkin, & Tiedje, 1985). Temporal variation of N_2O flux is not confined to sporadic hot moments. Despite first being identified over forty years ago (Denmead, Freney, & Simpson, 1979), it is only in the last few years that diurnal variation of N_2O flux has been more widely discussed (e.g. (Keane et al., 2018; Keane, Morrison, McNamara, & Ineson, 2019)). A literature review indicated that this phenomenon is much more

common than previously thought, predominantly characterised by large emissions during the day and lower fluxes at night (Wu et al., 2021), though the opposite has been reported elsewhere ((Keane et al., 2019; Shurpali et al., 2016)).

Chamber techniques are particularly useful for measuring at high spatial resolution, with an individual chamber typically covering < 1 m^2. A chamber is placed over the land or water surface, enclosing a known volume of air (headspace). Multiple measurements of N_2O concentration within the headspace are taken during the closure period, and the flux is calculated from the change in concentration over time, adjusted for the chamber area and volume. Deploying multiple chambers is an effective way to capture the spatial variation of a particular area and they are an excellent way to perform replicated experimental comparisons between treatments in the field. Early iterations of flux chambers were operated manually (Livingston, 1995): headspace samples were removed from a sampling port using a syringe and transferred to a pre-evacuated gas tight vessel (e.g. Exetainer). Samples could then be analysed using a GC in the lab. Incremental advances have been made in chamber design, incorporating features to reduce chamber artefacts on the observed fluxes (e.g. a vent to equalise pressure between the headspace and the atmosphere (L. K. Xu et al., 2006)), but perhaps the most important development is automation of chambers. Data obtained from manual chambers are limited by investigator hours available to sampling the chambers, the delay between sampling and the time taken to process each sample on a GC, which is usually several (5- 15) minutes per concentration measurement. Due to this, N_2O flux data from manual chambers tend to consist of measurements collected at less than daily frequencies. Through automation, multiple daily measurements may be collected from every chamber, and the data are then limited by the method of analysing the N_2O concentration. When deployed in conjunction with laser N_2O analysers, automated chambers are now capable of producing quasi-continuous N_2O flux data at high spatial resolution. This creates a challenge of scaling up measurements from a small spatial scale to the landscape scale and greater (Levy et al., 2022). To achieve reliable

extrapolation, a thorough understanding of the driving variables is necessary (see next section). There are many variations of automated chamber design, ranging from off-the-shelf systems to measure fluxes from the soil alone (Licor (Courtois et al., 2019) and Eosense (Ramlow, Foster, Del Grosso, & Cotrufo, 2019)), to larger chambers which have measured fluxes from intact soil-plant systems (e.g. Skyline (Keane et al., 2018) and Skybeam (Keane et al., 2019)). Due to the diversity in chamber designs, there have been concerted efforts in recent years to consolidate methodology to ensure comparability of data generated (Grace et al., 2020).

Micrometeorological methods operate at a spatial scale one to two orders of magnitude greater than chambers. Eddy covariance (EC) is the most common of several micrometeorological variations, including relaxed eddy accumulation and disjunct eddy covariation, which relate wind direction and gas concentration to infer fluxes (Leuning & Moncrieff, 1990). EC requires high frequency (> 1 Hz) measurements of wind velocity and gas concentration in turbulent air to model the integrated flux from a spatial area of hundreds to thousands of square metres, generally averaged over time periods of 30 minutes. The extent of the area measured (the system footprint or fetch) is a function of turbulence, wind velocity and the height of the sensing equipment above the vegetation canopy or land surface. Whilst EC has become a 'gold standard' approach for measuring ecosystem fluxes of the trace gases CO_2 and CH_4 (Gielen, 2017), its application to N_2O has been relatively less common, being restricted by the development of analytical equipment capable of the frequency required (see previous section). There are, however, limitations to EC and other micrometeorological techniques. Briefly, they require atmospheric turbulence above the boundary layer and during stable atmospheric conditions (often at night) it is not possible to model fluxes reliably in this way. Homogeneity of the measured landscape is also assumed, such as a forest canopy, grassland or monocropping, making EC unsuitable for mosaic ecosystems. Similarly, steep gradients in the terrain can cause advection, which also breaks the assumptions of EC mathematics. Finally, EC is a hostage to wind direction: commonly an EC tower will be sited to maximise it footprint

according to the prevailing wind. When the wind is not from this direction it cannot measure the study landscape. Due to the combination of these limitations, it is not uncommon for EC datasets to have gaps of around 50% of the study period. While robust mathematical models exist for gap filling ecosystem CO_2 fluxes based on solar radiation (photosynthesis) and temperature (respiration) (Wutzler et al., 2018), modelling N_2O is much more challenging (see other sections which discuss this). Other approaches which measure fluxes at similar scales include the gradient method and the box method (which uses a mass balance approach- measures the incoming and outgoing concentration to estimate net flux from a given volume), but these are much less frequently used.

Estimates of N_2O at a larger scale rely on moving platform (ship or aircraft) mounted applications of the described measurement technologies to monitor atmospheric concentrations (Desjardins, Brach, Alvo, & Schuepp, 1982). Optical approaches for measuring N_2O can be applied from satellites. However, despite the first data being produced in this way over 30 years ago (Chédin et al., 2002), there remain no openly available, globally validated, N_2O satellite data (Barret, Gouzenes, Le Flochmoen, & Ferrant, 2021). This is in stark contrast with the carbon GHGs, CO_2 and CH_4, which are continuously monitored on a global basis from the well-established space-borne platforms SCIAMACHY (Bergamaschi et al., 2009) and GOSAT (Turner et al., 2015).

3.3.2.3. Stable isotopes

Nitrogen (N) has two naturally occurring stable isotopes, ^{15}N and ^{14}N, with the lighter ^{14}N being by far the more abundant of the two (^{14}N represents > 99.6% of natural N). Any transformation of N compounds will undergo fractionation, where the lighter isotope is favoured, meaning that the product of a reaction will be depleted in ^{15}N relative to the substrate, provided that all the substrate is not exhausted (*i.e.* the reaction goes to completion). The ratio of the two stable isotopes, ^{15}N / ^{14}N ($\delta^{15}N$) in any nitrogenous compound is expressed in permille (‰). Naturally

occurring ^{15}N is referred to as natural abundance and can be a useful tool to infer what processes the compound has been subjected to. Enriching N compounds in ^{15}N, or 'labelling', enables experiments to trace N through biological systems. In this way, measuring the isotopic composition of N_2O ($\delta^{15}N_2O$) following the addition of a labelled substrate can help determine the source of the N_2O, whether that is a process or a location. Imagine a soil known to produce N_2O; if the $\delta^{15}N_2O$ increases following the application of a labelled nitrate compound $^{15}NO_3^-$, it could reasonably be inferred that the N_2O was the product of denitrification. Similarly, the stable heavy isotope of oxygen (^{18}O) has been used to demonstrate that the O atom incorporated in N_2O released from soils is predominantly derived from water rather than nitrate (Kool, Wrage, Oenema, Harris, & Van Groenigen, 2009).

Furthermore, since the two Nitrogen positions in N_2O are not equivalent ($N^-{=}N^+{=}O$), there are two locations at which ^{15}N may occur. This leads to the possibility of four distinct N isotopocules of N_2O:

1. $^{14}N^-{=}^{14}N^+{=}O$ M.W. 44
2. $^{15}N^-{=}^{14}N^+{=}O$ M.W. 45
3. $^{14}N^-{=}^{15}N^+{=}O$ M.W. 45
4. $^{15}N^-{=}^{15}N^+{=}O$ M.W. 46

The two positions occupied by N atoms are designated the labels alpha (α), which is the end position and beta (β) the central position, and the difference between the $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ is known as the site preference (SP) (Yoshida & Toyoda, 2000). As with fractionation, SP is known to differ with natural reactions, and as such has been suggested to be capable of distinguishing N_2O produced from nitrification and denitrification (Ostrom et al., 2007). In this way, stable isotopes represent a powerful non-destructive tool for unravelling nitrogen cycling processes governing N_2O emissions. This application of stable isotopes represents an advance on the acetylene block technique (Davidson & Swank, 1987), which utilises the inhibitory action of

acetylene on nitrifiers' ammonium monooxygenase enzyme to differentiate between N_2O derived from nitrification and denitrification; despite evidence that this technique underestimates denitrification (Bollmann & Conrad, 1997) it is still used quite widely (Qiu et al., 2024). A further step in the use of stable isotopes has been the development of the $^{15}N_2O$ pool dilution technique ($^{15}N_2OPD$) which quantifies N_2O uptake from the net N_2O flux even when this is positive (Wen et al., 2016), and has been used to investigate N_2O dynamics at different depths in intact soil profiles (Button et al., 2023). Typically, stable isotopes have been measured using IRMS, by detecting the mass to charge ratio of a molecule (see previous section). However, the standard configuration of IRMS is insufficient to discriminate between isotopocules of N_2O where ^{15}N occupies either the α or β position. Laser analysers, however, have been shown to be particularly adept at measuring this, which has proven to be invaluable in this area of research.

3.3.3. Regulation

An important feature of Stuart Ferguson's work was to emphasise the significance of control of respiratory processes at a metabolic level, rather than just at the control of the production of the relevant enzymatic gene products. The net production or removal of nitrous oxide in a given setting depends on the expression of enzymes that generate and/or remove nitrous oxide, but also, as these are redox processes, the factors governing the flow of electrons to substrates of these enzymes. Given that nitrous oxide is a freely diffusible gas, the site of production need not be in the same cell as the site of removal.

Unlike regulation in many other biological systems, expression of *nosZ* appears not to be controlled in response to the concentration of either its substrate (N_2O) nor its product (N_2), both of which are rather inert. Instead, gene expression is regulated by proxy measurements, that may be relevant to the physiology of the organism carrying out the nitrous oxide reduction activity. In the model denitrifier *Paracoccus denitrificans*, for example, *nosZ* transcription is activated dually by lack of oxygen (via FnrP) and nitric oxide (via NNR) (Bergaust, van Spanning,

Frostegard, & Bakken, 2012), leading to production of NosZ when denitrification is required for activity, and when an earlier intermediate in the pathway is abundant. Detailed understanding of the regulation of *nosZ* expression is limited to a small number of model organisms, and the extent to which this is in some sense representative of N_2O-reducing microbes as a whole is unclear. Some studies of *nosZ* transcription *in situ* in soils have been carried out, and shifts in expression observed (*e.g.* in response to treatments such as biochar (Kim et al., 2020)), but whether the architecture of the underlying regulatory circuits is similar between diverse organisms is unknown. The clade II NosZ-containing organisms are often non-denitirifiers, and so the cues that are sensed to control nosZ expression are likely to be different from model denitrifier organisms. To date, there has been a lack of biochemical characterisation of regulation in these strains.

N_2O can be generated via NO reductase in denitrifiers or nitrifier-denitrifiers, and a by-product of other N-cycling enzymes, *e.g.* hydroxylamine oxidase. Regulation of these processes varies but abundance of nitrate, nitrite, nitric oxide and oxygen are all cues of importance.

At a cellular level, even once the enzymes are produced, their activity depends on the flow of electrons towards the substrates. The N_2O/N_2 couple has a very high redox potential (+1355 mV) and so this is thermodynamically very favourable. This is offset by the very small ratio of N_2O to N_2 in most environments. N_2 is less soluble in aqueous systems than N_2O, but its very high atmospheric concentration (800,000 ppm) compared to typical N_2O concentration (in the order of 1 ppm) makes the effective redox potential for N_2O lower than the standard $E^{o'}$, but still c. +1V, and thus entirely satisfactory in terms of electron flow to this substrate. At the same time, the redox potential couple for NO/N_2O is also high compared to O_2/H_2O and NO_3^-/NO_2^- and NO_2^-/NO, so the production of N_2O is also favourable energetically. At steady state, the residual concentration of N_2O will depend on the ammonium and nitrate content of the system (which governs the rate of production of NO, and thence N_2O). The magnitude of this N_2O

concentration will vary according to the underlying physiological properties of the microbes themselves (the expression of N cycling genes, the organisation of respiratory chains that will differently affect the relative favourability of electron flow to particular reductases according to how tightly coupled these are to pmf generation).

The above experimental approaches have explored regulation at a population level, averaged across billions of bacterial cells. At the level of individual cells in a population of *P. denitrificans*, it appears that a bet-hedging approach is taken, with stochastic expression of nitrite reductase, but constant low level expression of nitrous oxide reductase. It is proposed that this strategy protects *Paracoccus* during the transition to anoxia, as cells can survive by scavenging nitrous oxide (which is a freely diffusible intermediate). This regulatory strategy indicates a particular selection pressure for retaining nitrous oxide reductase, in environments undergoing transition from aerobic, microoxic to anoxic conditions (Lycus et al., 2018).

3.3.4. Biochemistry

The biochemical properties of nitrous oxide reductase, relevant to electron transfer and enzymology have been reviewed in detail by Hein and Simon (Hein & Simon, 2019). The affinity of NosZ for nitrous oxide is a key factor, covered already above under the section on Nos I and II. The copper centre of the nitrous oxide reductase is the source of sensitivity by molecular oxygen and the enzyme is also significantly inhibited at low pH. These biochemical features are significant drivers of nitrous oxide production in natural and engineered systems.

In soil systems, nitrous oxide removal activity is typically inhibited at lower pH (progressively from c. pH 6.8) (Henault et al., 2019). It appears that the effect of low pH on nitrous oxide reduction occurs post-transcriptionally, as *nosZ* expression at a transcript level is not inhibited by pH (Frostegard, Vick, Lim, Bakken, & Shapleigh, 2022). Fujita and Dooley (2007) (Fujita & Dooley, 2007) demonstrated that nitrous oxide reductase could be activated reductively, and that doing so at different pHs led to distinct pH activity profiles. In each case, it

was observed that the activity of N_2O reductase declined significantly when assayed below pH 7 (even when activated by treatment at pH 5.7). Carreira and coworkers showed that the catalytic Cu_Z centre of N_2O reductase from *Marinobacter hydrocarbonoclasticus* is mainly in a 4Cu1S from when assembled at pH 6.5 but 4Cu2S at pH 7.5 or 8.5 (Carreira, Nunes, Mestre, Moura, & Pauleta, 2020). That in vivo additional levels of regulation exist to maintain nitrous oxide reductase activity is supported by observations of N_2O reduction at low pH, such as in the recent report of sustained, growth-linked nitrous oxide reduction at pH 4.5 (He et al., 2024).

The Cu_z site of nitrous oxide reductase can occupy a variety of spectroscopic and structural forms, and this can depend on the oxygen status during the preparation of the enzyme (Wust et al., 2012). Whilst the nitrous oxide reductase appears sensitive to oxygen, N_2O reduction can continue in some organisms under aerobic conditions, and the associated N_2O reductase retains *in vitro* activity (Bell & Ferguson, 1991; Berks, Baratta, Richardson, & Ferguson, 1993). In recent work, Wang classified nitrous oxide-reducing systems into sensitive, tolerant and intolerant, arguing that the differences in tolerance do not sit at the level of the enzyme itself, but the cellular environment in which nitrous oxide reductase may be protected by oxygen scavenging systems (Z. Wang et al., 2023).

Another way in which inhibition may be important for consideration of overall N_2O fluxes relates to the availability of copper for nitrous oxide reductase. Insufficient availability would render the enzyme inactive (Sullivan et al., 2013). In complex microbial communities, the scavenging of copper, *e.g.* through the release of copper-binding methanobactins is reported to influence denitrification including nitrous oxide reduction (Chang et al., 2021; Chang et al., 2023).

4. Engineering solutions to the GHG emission crisis

Better implementation of effective nitrogen management practices (using existing technological innovations), along with behavioural change towards more sustainable diets, is predicted to be able to deliver a 40% reduction in N_2O emissions by 2050 (UNEP report). For the final part of this article, we ask -how can we extend this approach to climate change mitigation still further, with technologies developed based on the underlying microbial biochemistry of nitrous oxide transformation?

4.1. Behavioural change

Bold, creative and inspirational ideas for technologies have the potential to transform nitrous oxide emissions (Stein, 2024). That said, implementation of existing and new technologies to reduce nitrous oxide emissions will rely on engagement and behavioural change. In order to ensure that the global challenge is met by local and regional action needs to take into account the cultural heritage and local knowledge of farmers, land-owners and other relevant stakeholders and organisations, as well as international scientific analysis (see e.g. (Shakoor et al., 2024)). We know that imposed technological solutions can fail to work, if the end users are not made active participants, whose knowledge is respected (Ensor, Johnson, Vorbach, & Moir, 2025). A process in which end user / stakeholders and scientist / policy implementers engage to discuss the challenges and suitable technological solutions can be enriched though demonstrating the scientific underpinnings, such as previously unfamiliar and “invisible” concepts in microbiology (Ensor et al., 2025). The sharing of knowledge about underlying processes and how this relates to land or water-course management and approaches to mitigate climate change could be the basis for technology co-design to support grounded technologies, more likely to lead to engaged parties and positive outcomes.

4.2. Microbial variables as decision-making tools

Measuring molecular indicators related to nitrous oxide emissions has the potential to integrate the ecosystem scale with the underlying processes involved in nitrous oxide formation and consumption, a key challenge in the field (Butterbach-Bahl et al., 2013). As discussed earlier in the chapter, we know that microbial molecular measurements enrich our power to predict nitrous oxide emissions (Frostegard et al., 2022; Han et al., 2024; Hao et al., 2022; Liu et al., 2022). Such studies provide insight, for a given setting *e.g.* either a type of environment (river, lake, soil, digestor) or an example of that type (different crops in different agricultural land in different places, at different times), of the dominant processes pertinent to nitrous oxide emissions, which can drive management decisions (e.g. application of nitrification inhibitors (Recio, Alvarez, Rodriguez-Quijano, & Vallejo, 2019; Yin, Gao, Kuang, & Zhang, 2023)). Such studies allow us to make broad conclusions about management strategies (like the use of no-till or minimum tillage strategies); however, local decision-making requires developing what are essentially research tools for use in agricultural management. Sequencing technologies continue to become progressively less expensive year-on-year, and we need to consider what a sequencing-type technology application might be like, that would fit with the roles of end-users who make a living from the land, are producers of food, and stewards of our landscapes. Any technology would depend on the timescale over which it would be needed -e.g. making annual assessments to budget fertilizer application based on projected productivity and nitrogen use efficiency and GHG emissions, versus in-season assessments. Similarly, what would the spatial scale of analysis need to be (noting that relevant measures can vary over very short distance scales (Giles, Morley, Baggs, & Daniell, 2012), but, microbial measurements at spatial scales relevant to agricultural practice have potential (Enwall, Throbäck, Stenberg, Söderström, & Hallin, 2010) but we are still some way off having robust tools for use by farmers to reduce N_2O emissions.

4.3. Biological interventions

Two approaches to be considered are the engineering of organisms themselves, and then considering the introduction of these, or existing organisms or using other modes of control to influence the community structure and function, relevant to nitrous oxide release.

4.3.1. Engineering organisms

Given the abundance of nitrous oxide released in soils in agricultural land, it is worth considering the development of genetically modified plants that are able to take up and remove nitrous oxide. The N_2O reductase requires complex assembly factors, but we have a fair understanding of the underlying genetic and biochemical features of this. Nitrous oxide reductase from *Pseudomonas stutzeri* has been introduced transgenically into tobacco, and expressed selectively in root tissues (Wan, Johnson, & Altosaar, 2012). The resultant strains were shown to have nitrous oxide reductase using a non-physiological electron donor (methyl viologen), and an essential next step would be to link the nitrous oxide reductase to an operational respiratory chain. The potential to explore the use of other *nosZ* genes, whose protein products could have higher affinity for N_2O (such as members of clade II) would be of interest to explore, as well as expressing the enzyme in leaves as well as roots, with the aim of such plants scavenging N_2O released into the atmosphere.

Engineering bacterial strains to have a set of beneficial traits relating to nitrous oxide removal would also be valuable for a range of settings. Nitrous oxide reductase gene clusters could be introduced into commonly highly abundant (e.g. soil) bacteria to supplement the nitrous oxide-reducing capacity of soils or other environments, as well as aiming to engineer improvements such as high expression, high affinity, and oxygen tolerance (most likely through co-expression with oxygen and ROS scavenging systems), or through engineering in copper capturing small methanobactin production to protect nitrous oxide reductase from copper limitation (Chang et al., 2023).

The ethical, regulatory and societal challenges of introducing technologies based on these approaches should not be underestimated, and very careful consideration of these would need to be conducted to take forward these engineering approaches. Any responsible engineering biology approach would require a deep analysis of the potential ramifications of introduced engineered organisms on health and the environment.

4.3.2. Engineering communities

Without introducing engineered organisms, we potentially can make a significant impact on nitrous oxide release through influencing the microbial community composition in a given setting. This might be through actively monitoring microbial community composition and function and implementing a relevant management approach (for example, altering pH (Henault et al., 2019) or control of carbon source availability (Qi et al., 2022)) or, on the other hand, by inoculating particular biological agents into the system. This might be via seed treatments or leaf sprays with endophytic bacteria, for example, where a founder effect can increase the abundance of introduced microorganisms for a substantial period. Mycorrhizae can enhance the effectiveness of bacterial nitrous oxide removal (X. Li et al., 2023). Direct inoculation with known nitrous oxide removing dentrifier *Pseudomonas stutzeri* was shown to support nitrous oxide removal in soil (Gao et al., 2024). Easy wins would include ensuring that inoculants used with major agricultural crops have not lost their ancestral nitrous oxide reductase capability, as was found to be the case for some commercial alfalfa inoculants (Brambilla, Frare, Soto, Jozefkowicz, & Ayub, 2018).

Successful implementation of such community engineering technologies will be reliant on routine monitoring to gain feedback on the success or otherwise of inoculation strategies on microbial community composition and nitrous oxide removal.

4.4. Better monitoring and modelling

Missing biochemistry and understanding of microbial physiology limits the accuracy and predictability of current global nitrous oxide emission models. A deeper and broader understanding of how nitrous oxide is processed in different organisms and the cellular apparatuses that are involved in affording some resistance to oxygen is required, work that includes more fundamental biochemical characterisation especially of the clade II NosZ. Allied to this, there is a challenge with nitrous oxide measurement at both ends of the spatial scale - the absence of global N_2O satellite data sets on one end, and the lack of simple and inexpensive monitoring systems for N_2O by users rather than researchers at the other end. The potential to develop nitrous oxide monitoring based on N_2O biology should be more seriously elaborated, based on NosZ and its unique Cu_Z cofactor, as a target. The potential to develop biosensors to act as passive monitoring systems to measure an integrated signal of N_2O accumulation can be conceived of based on the competitive advantage of *nosZ*-containing strains versus isogenic *nosZ* deficient variants.

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6. Dedication

JM dedicates this chapter to Prof. Stuart Ferguson: supervisor, mentor, and inspiration.

I was lucky enough to be taken on by Stuart for a D. Phil at the beginning of the 1990s. His intellectual authority, wisdom and wit provided me with the guide I needed to develop as a scientist. His distinctive hands-off style allowed me freedom to roam and explore the biological system in the company of other great colleagues in Stuart's lab around that time, notably David Richardson, Ben Berks, Al McEwan and Dudley Page. The work I undertook then was the springboard to an academic career, and lifelong love of microbial biochemistry and microbial physiology. As an independent scientist, I remained connected to Stuart's work and his thoughtful and deep view of biochemistry was always influential. As an academic, Stuart was also a powerful educator as well as researcher, and his influence on me as an educator was also significant, and I know that his approach, values and views on that have impacted on my own in a resonant way. Whilst the tone of this chapter may lean more towards the applied than was Stuart's tendency, the need to continually assess and re-assess the underpinning biological mechanisms is rooted in Stuart's science world view in the way he showed us.

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Figure1: Nitrogen cycle schema illustrating dominant routes for generation and removal of nitrous oxide.

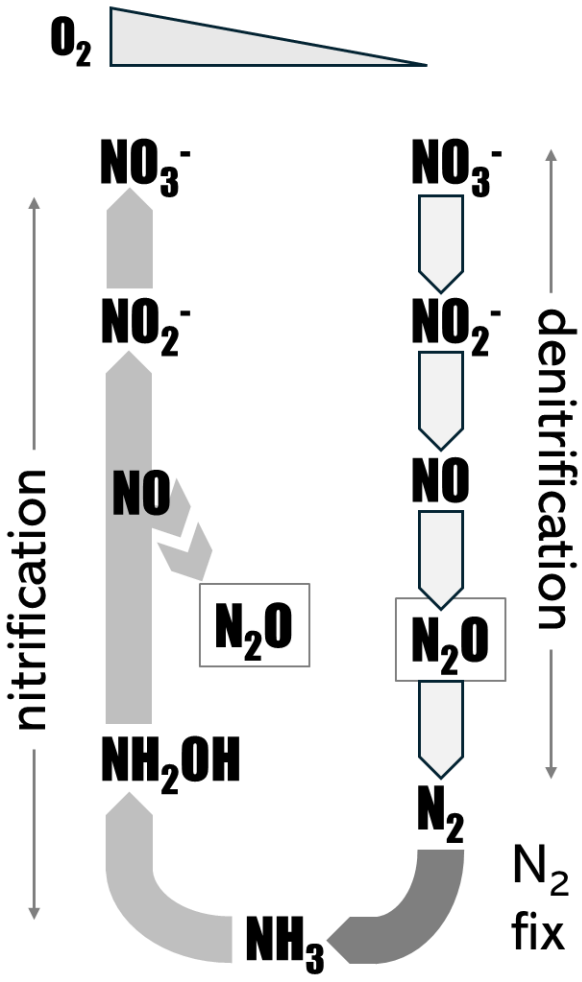

Figure 2. Predicted fold for *C. fetus* NosZ (clade II) determined using alphafold3 (pink/dark grey) aligned with the experimentally determined structure of NosZ from *P. denitrificans* (green/pale grey) with the Cu_Z active site shown as spheres with copper (orange) and sulfur (yellow) (pale grey in the print copy).

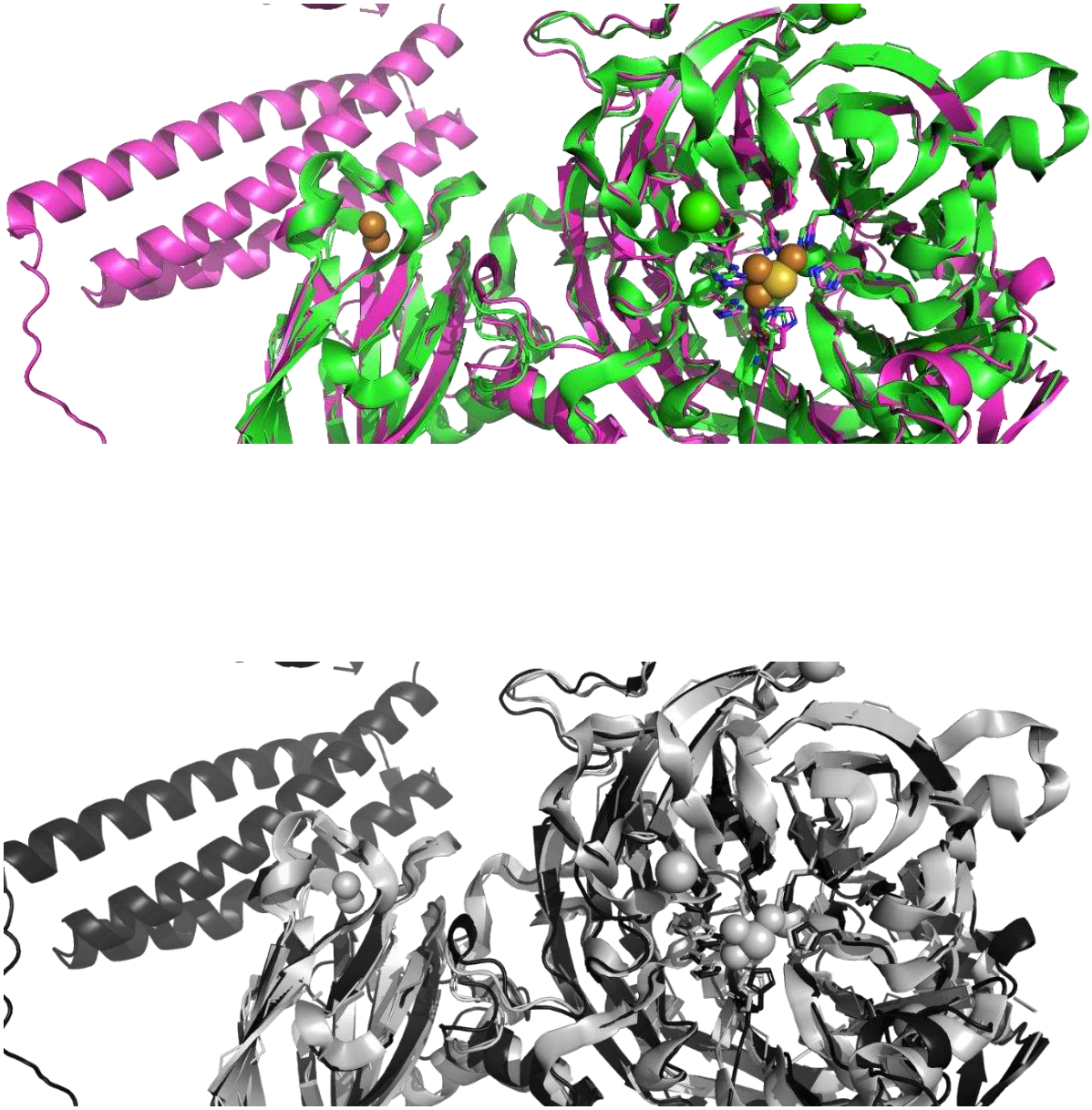

Figure 3. N_2O measurement at different scales. Spatial and temporal scales of the upscaling challenges, from measurements using chambers and eddy covariance which cover small scales, to national-scale annual fluxes. The bars show the typical resolution of the different measurement techniques. The numbers respond to the challenges: 1. Quantifying uncertainty in spatial upscaling of chamber fluxes to field scale; 2. Quantifying uncertainty in temporal upscaling of chamber fluxes to annual scale; 3. Reducing uncertainty in spatial and temporal upscaling of chamber fluxes via improved instrumentation; 4. Quantifying uncertainty in eddy covariance measurements of field scale fluxes; 5. Quantifying aggregation error in spatial upscaling (from Figure 1. in Levy et al., 2022).