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Complete List of Authors:	Lehnert, Nicolai; University of Michigan, Department of Chemistry Musselman, Bradley; University of Michigan, Department of Chemistry Seefeldt, Lance; Utah State University, Chemistry and Biochemistry

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Viewpoint Article

Grand Challenges in the Nitrogen Cycle

Nicolai Lehnert,[1*] Bradley W. Musselman,[1] Lance Seefeldt[2*]

[1]Department of Chemistry and Department of Biophysics, The University of Michigan, Ann Arbor, MI 48109-1055
[2]Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0305

Overview. The Nitrogen Cycle (N Cycle) is one of the most important biogeochemical cycles on earth, as all life forms are dependent on nitrogen.[1, 2] Although the Carbon Cycle usually receives most attention in the media, due to the looming climate crisis caused by anthropogenic carbon dioxide (CO_2) emissions, it is in fact the N Cycle that is most affected by human activities.[3] Nitrogen is abundant in the air in the form of gaseous dinitrogen (N_2), but it cannot be readily taken up by plants. For plants to access nitrogen, N_2 must first be “fixed,” i.e. converted into other forms, especially ammonia (NH_3; NH_4^+ at physiological pH). Natural processes deliver around 120 megatons per year of bioavailable nitrogen to the biosphere,[4] mostly due to nitrogen fixation by microbes, with some in association with leguminous plants, like alfalfa, clover, beans, peas, lentils and lupins. Humans have greatly augmented these processes, using the industrial Haber-Bosch process, where N_2 and H_2 are converted to ammonia at high temperature and pressure, following the reaction: $N_2 + 3\ H_2 \rightarrow 2\ NH_3$. The Haber-Bosch process contributes at least the same amount of fixed nitrogen to the biosphere as do natural processes, roughly 100 million tons of nitrogen fertilizer each year. The Haber-Bosch process is responsible for around 2-3% of global fossil fuel consumption.[4] Importantly, current estimates indicate that around 40% of the human population depend on the human contribution to the nitrogen cycle.[4, 5] In the developed world, where nitrogen-containing fertilizer is readily available, farmers routinely overfertilize their fields to ensure that nitrogen is never a limiting nutrient. However, only about 30 - 50% of this nitrogen is taken up (assimilated) by crops. The rest is converted between various forms of nitrogen oxides (termed “NO_x”) by soil microbes, as shown in Figure 1. Here, ammonia is stepwise oxidized to nitrate (NO_3^-) in the nitrification process, with hydroxylamine (NH_2OH), nitric oxide (NO) and nitrite (NO_2^-) as obligate intermediates.[6] The higher nitrogen oxides produced in this way are then further metabolized in the

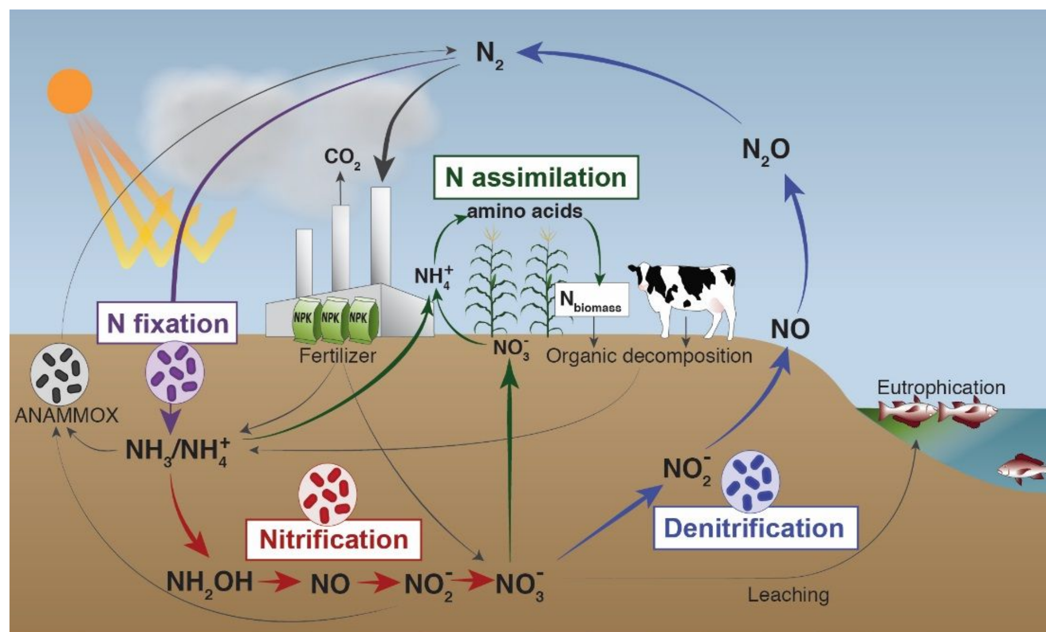


Figure 1. Overview of the Nitrogen Cycle, highlighting the most important pathways and critical intermediates of the different interconversions of nitrogen in the environment.

denitrification process, and reduced back down to nitrous oxide (N_2O) and N_2 as the final products. Dissimilatory reduction of nitrate to ammonia and the ANAMMOX process are additional microbial pathways, although scientists are unsure how much these processes contribute to total nitrogen cycling in soils.

Nitrification and denitrification lead to multiple environmental problems, because major products of these processes readily leach from the soil into the water or escape into the atmosphere. The most severe problems are nitrate runoff into waterways and release of N_2O, a potent greenhouse gas and ozone depleting agent.[7] Nitrate emitted from agricultural and industrial processes is now considered one of the world's most prevalent wastewater pollutants, and its treatment is recognized as a major challenge.[8] Massive "dead zones" now occur annually in many of the world's major water bodies due to the accumulated effects of nitrate (as well as phosphate) runoff, causing cyanobacteria and algae blooms that can deoxygenate large volumes of water. Nitrous oxide is a major contributor to the destruction of the ozone layer, and it has a 100-year greenhouse warming potential nearly 300 times higher than CO_2. Globally, N_2O traps more radiation than any anthropogenic greenhouse gas other than carbon dioxide (CO_2) and methane (CH_4). Around 75% of all anthropogenic N_2O emissions originate from agricultural soil and result from (over)fertilization.

Besides these big environmental issues related to the N cycle and the ever-growing human population of the planet,[9] there are many basic scientific questions

surrounding the different processes in the N cycle (see Figure 1) that, in our view, are still only poorly understood. These are further discussed in the following paragraphs.

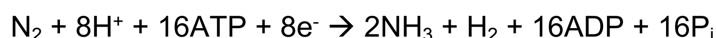
Biological and Synthetic Nitrogen Fixation.

The fixation of dinitrogen (N_2) to ammonia (NH_3) is a six-electron/six-proton reduction reaction that is overall exergonic, yet is vanishingly slow without the addition of significant amounts of energy and/or the action of an effective catalyst.[10] The difficulty in traversing the multi-step reaction landscape from N_2 to $2NH_3$ arises from the inertness of the N≡N triple bond, which results in a high energy of activation to reach the key intermediates. The industrial Haber-Bosch process achieves this challenging reaction by using H_2 as the source of electrons and protons, and using high temperatures to impart sufficient energy for reactants to scale the considerable activation energy, and the inclusion of a Fe-based catalyst.[10] The H_2 for this reaction is most often generated from fossil fuels, often through reforming of methane, which accounts for the majority of the fossil fuel needed for this process. The heat required is also a significant energy input, most often deriving from burning fossil fuels. The reaction is also conducted at high pressures to favor product NH_3 formation, with an energy demand to achieve these high pressures. As human civilization is striving to reduce fossil fuel consumption, it will be important to make changes to the Haber-Bosch process as well. In particular, the required, large amounts of H_2 should be produced from water splitting, powered by solar energy, and the heat and pressure could be derived from renewable energy sources like solar, wind, and tides. The slow conversion from fossil fuels to renewable energy to drive this reaction arises from the abundance and low costs for fossil fuels, the lack of building in actual costs for CO_2 impact, and the need for more efficient catalysts for water splitting. For decentralized ammonia production, especially in rural areas and developing countries, electrocatalytic nitrogen reduction is a vital alternative to large-scale Haber-Bosch plants in our opinion, especially when powered by solar energy. In particular, within this process, H_2 gas is no longer needed, and instead, water serves as the source for electrons and protons. This is a new, exciting area of research that, in our opinion, has the potential to make a real change in the way that ammonia could be produced at scale in the future.

Biological nitrogen fixation predates the Haber-Bosch process by about 2 billion years. The biological reaction occurs in a wide range of bacteria and some archaea, which collectively are referred to as diazotrophs. Nitrogen fixation is not known in any

eukaryote, although some eukaryotes have evolved symbiotic relationships with bacteria that provide N to the host (e.g., soybean-bacteria symbiosis). As an alternative to industrial nitrogen fixation, there is a great current effort to clone nitrogen fixation genes into crops, which would enable plants to fix their own nitrogen, and in this way, circumvent the problem of the (over)usage of synthetic fertilizer and the pollution that it causes. Although still in an early stage, we believe that there is great potential in this approach, and research in this area should therefore be accelerated.

The enzyme that catalyzes N_2 fixation is called nitrogenase, of which there are three known isozymes named for the metal that is part of their active site: Mo-, V-, Fe-nitrogenase. The three isozymes are coded by different genes (called *nif, vnf, and anf*), and thus each isozyme is composed of unique polypeptides. While each isozyme is unique, they all share a similar architecture and metal clusters. While nitrogenase and the Haber-Bosch reactions both use Fe, these two processes achieve N_2 reduction to NH_3 in fundamentally different ways. Nitrogenases utilized electrons derived from metabolism and protons derived from water.[11] The energy for the nitrogenase reaction comes from the hydrolysis of ATP to ADP and Pi. In contrast to the Haber-Bosch process, nitrogenases yield H_2 rather than consume H_2.



$$N_2 + 8H^+ + 16ATP + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Further, nitrogenase functions at ambient temperature and pressure. Driven in part by a goal of gaining insights into how Mother Nature achieves this difficult reaction under benign conditions, there has been a considerable effort over decades exploring mechanistic details of the nitrogenase reaction mechanism.[11] The reaction is complex and involves many different cofactors, proteins, large scale dynamics, and the hydrolysis of ATP to ADP and P_i, and, in our opinion, is far from being understood. progress has been made in many aspects of this complex array of events.

Among the most recent insights is the revelation that the release of H_2 is mechanistically coupled to the binding and activation of N_2. The active site of the Mo-nitrogenase, called FeMo-cofactor, is where N_2 binds and is reduced. While far from settled, there is good evidence now, in our opinion, that this cofactor accumulates 4 electrons and 4 protons as two metal-bound hydrides and two protons bound to cofactor sulfides (Figure 2). Several different approaches have suggested that accumulation of electrons on the FeMo-cofactor results in ligation changes within the cofactor, including breaking of Fe-S bonds to accommodate the binding of substrates.[12] This activated state of the cofactor, called E_4(4H), is charged for N_2 binding. In what is proposed to be a

concerted process, the two hydrides combine in a process called reductive elimination to form H_2, with the concomitant binding of N_2.[11] The release of H_2, an exergonic process, is proposed to be coupled to the endergonic binding of N_2, resulting in an overall exergonic event. Once N_2 is bound, the two electrons left in the cofactor from the reductive elimination reaction are proposed to convert the metal-bound N_2 to a metal-bound diazenido (HN=NH) intermediate. Experimental findings coupled with theory all point to a shallow reaction barrier for this essential step in the reaction, with the equilibrium being readily reversible.[13] The profound lowering of what is expected to be a substantial activation barrier at this step in the reaction lies at the heart of what makes nitrogenase such an excellent catalyst for this challenging reaction. Subsequent reduction of the bound diazenido-metal species by 4 more electrons results in the release of $2NH_3$. While there are many details of this process that remain to be resolved, it appears that some of the key aspects of the reaction are coming into view. We think that the parallels in small molecule activation by nitrogenase and organometallic catalysts, via the key reductive elimination step described above, is a particularly fascinating aspect of this new, mechanistic proposal.

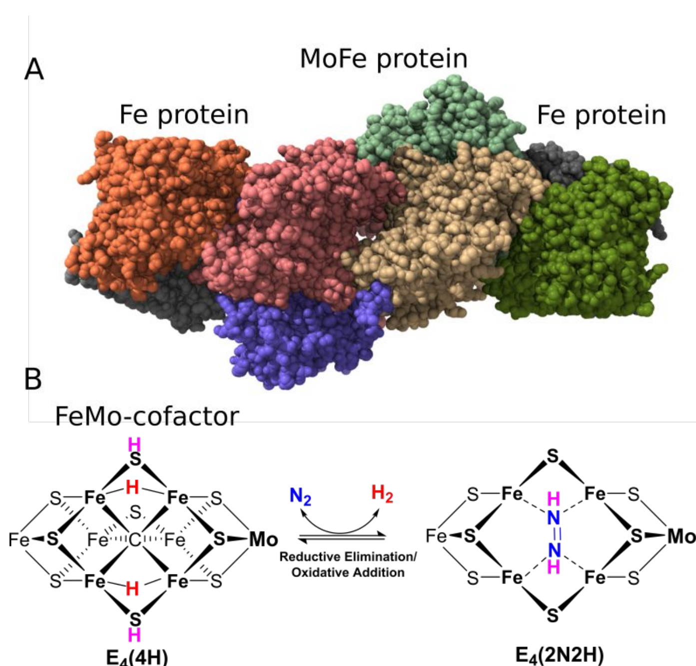


Figure 2. Mo-nitrogenase and its active site FeMo-cofactor. Panel A: Nitrogenase complex composed of one MoFe protein comprised of two αβ pairs (pink/magenta and tan/teel) and two dimeric Fe proteins (green/gray and orange/gray). Generated from PDB 1N2C using ChimeraX 1.0. Panel B: One active-site FeMo-cofactor is located in each αβ pair of the MoFe protein. Shown is the proposed structure of the 4 electron/proton reduced E_4(4H) state (left) with two bound hydrides (red) and two protons (pink). Reductive elimination of H_2 and binding of N_2 results in the E_4(2N2H) state shown on the right with N shown in blue. The oxidative addition of H_2 reverses the equilibrium. The central atoms of the FeMo-cofactor are omitted on the right for clarity.

There has been good progress in developing both homogeneous and heterogeneous catalysts that can achieve N_2 reduction to ammonia.[14] Early successes were with mononuclear Mo-based homogeneous complexes. While the discovery of N_2 reduction to NH_3 by these complexes was a breakthrough, it was also clear that the catalysts were slow and had low turnover numbers. The turnover number for these complexes of less than 100 contrasts with a turnover number of over 10^6 for nitrogenase. Many studies have followed these early discoveries, expanding the types of metals and the complexity of the metal complexes that can achieve N_2 reduction. While there has been an improvement in both the turnover frequency and number for many of these complexes, we believe that significant progress is still needed to reach activity values with these catalysts that would actually make them competitive with the Haber-Bosch or nitrogenase catalysts. It seems unlikely to us that these catalysts will reach a stage that

they could be applied at scale. Instead, the great benefit of studies of these catalysts lies in the elucidation of underlying mechanistic principles in nitrogen fixation that should drive the design of ever-better catalysts and sharpen our understanding of the mechanism of nitrogenase itself.

Heterogeneous catalysts offer some significant advantages for many chemical reactions, and there have been considerable efforts to design heterogeneous catalysts aside from the Haber-Bosch catalyst to achieve N_2 reduction to NH_3. The pace of reports of heterogeneous catalysts for N_2 reduction has been accelerating over the last few years. Many of these efforts seek to drive the reaction electrochemically or with light, thus offering an obvious pathway to a greener reaction, as mentioned above. The turnover frequency, number, and electron use efficiency for some catalysts are indeed impressive. One of the major challenges that we see in this field is the difficulty in directly comparing activity descriptors among catalysts, because of the challenge of quantifying the amount of catalyst and the lack of a uniform set of units for reporting rates. A further challenge for all studies on N_2 reduction lies in the perils of accurately quantifying NH_3. These are major challenges, in our opinion, that slow down the rate at which better catalysts can be discovered. A recent review documents these challenges, and provides suggestions for using $^{15}N_2$ to provide accurate N_2 reduction numbers.[15] We expect consolidation of many studies over the coming years, as more reliable NH_3 detection approaches will be widely adopted.

What is already clear is that tremendous progress is being made in the design of better homogeneous and heterogeneous catalysts for N_2 reduction. The advancement of robust theory seems ready to be applied to the design of more robust catalysts. The interplay of insights into the details of the enzyme, application of theory, complex syntheses, and accurate product measurements should be a powerful driver in the production of superior catalysts going forward.

Enzymes in Nitrification, Denitrification and other Processes.

The chemistry of the N cycle comprises many unique (and sometimes surprising) reactions, unusual enzymes, and cofactors with unusual properties, and there remains much to be discovered. A major challenge in many of the pathways is the lack of proper biochemical characterization of key enzymes, which hampers our progress in understanding these pathways and the mechanisms of the involved enzymes (see below for details). In our opinion, overcoming this limitation should be a key focus of research

efforts in this field going forward. In addition, there is a need for model complex studies on some of the key reactions present in these pathways, in order to better define the underlying chemical reactivity landscape that these enzymes operate in. An additional benefit of acquiring a detailed mechanistic understanding of the transformations in the N cycle is that these reactions are highly relevant for the clean and controlled cycling of nitrogen-based compounds in fuel cells.

Nitrification, the oxidation of ammonium to nitrite and nitrate, is the main process that connects biologically reactive nitrogen to other processes in the N cycle.[2, 6] Nitrification comprises ammonia-oxidizing bacteria and archae (AOB and AOA), which are responsible for the six-electron oxidation of ammonia to nitrite, and nitrite-oxidizing bacteria (NOB) that mediate its two-electron oxidation to nitrate. More recently, "complete ammonia-oxidizing" (COMAMMOX) bacteria, which affect the complete (eight electron) oxidation of ammonia to nitrate, were also identified. Importantly, microbial nitrification is the main process that competes with plants for ammonium fertilizer, and that determines the balance of ammonium and NO_x species available in the soil, for assimilation into plants and metabolism by other microbes (although more research is needed to determine how nitrification activity in the soil is impacted by environmental factors like rainfall, temperature, and availability of other nutrients in the soil). Because of this, nitrification inhibitors (for example, nitrapyrin) are currently used in agriculture to increase the life time ammonia in the soil, by shutting down nitrification. However, we view this practice as being problematic, as the environmental impacts of the compounds used for this purpose are often not clear. In our opinion, a better way to address this challenge is to stimulate the DNRA pathway instead (see below). However, currently it is not known how to do accomplish this, requiring more research into the ecology of the relevant microbes.

Nitrification is initiated by the oxidation (hydroxylation) of ammonia to hydroxylamine, catalyzed by Ammonia Monooxygenase (AMO). This enzyme is a member of the copper-containing, membrane-bound monooxygenase family that also includes particulate methane monooxygenase. AMO has largely defied expression and purification, and beyond substrate scope, little mechanistic information is available. The hydroxylamine product of AMO is then further oxidized by Hydroxylamine Oxidoreductase (HAO), which is a homotrimeric enzyme with eight *c*-type hemes per subunit. The active site heme, called "P460" (because of its Soret band at 460 nm in the reduced state), features an unusual double crosslink between a Tyr residue and the 5'

meso-carbon and an adjacent pyrrole α-carbon of the heme. However, the function of this crosslink for catalysis is not known. Because of the seven electron transfer hemes in HAO, mechanistic information for this enzyme is hard to obtain. In fact, for the last three decades, scientists had assumed that the product of HAO is nitrite (based on activity assays), originating from the four-electron oxidation of NH_2OH. Only recently were scientists able to show that HAO actually produces NO in a three-electron process. This further underlines our notion that nitrification in particular is in great need for more research activity, to determine even the most basic workings of this pathway. In fact, with the new discovery that HAO makes NO as the product, nitrification is now missing one enzyme, responsible for the oxidation of NO to nitrite. Candidates for a corresponding NO Oxidoreductase (NOO) are copper proteins, for example bidirectional copper nitrite reductases or the unusual Type I copper protein nitrosocyanin,[6] which has an usual square-pyramidal Cu site with His_2/Cys/Glu/H_2O ligation, indicating that it likely has a different function than other Type I copper proteins.

Although ammonia- and nitrite-oxidizing bacteria were isolated more than 125 years ago, the enzymology and physiology of these microbes remained largely mysterious (see ref. 6 for a detailed discussion). From our perspective, based on the status of the field, progress on nitrification is needed on many fronts, especially with respect to the isolation, expression, and purification of the key enzymes from different organisms to explore the biodiversity of these enzymes. Nitrifying microbes are difficult to cultivate in the laboratory and grow on a scale that would allow for deeper biochemical studies. On top of this, many of the key enzymes involved in nitrification are incompatible with standard recombinant DNA technology, and hence, difficult to obtain in suitable quantities. Another key question is the identification of the missing NOO. Finally, more studies into the molecular mechanisms of the nitrification enzymes are needed, using kinetic, spectroscopic, theoretical, and model complex studies. The latter will help to test mechanistic hypotheses and explore the basic chemistry that these types of active sites could mediate.

There are two major pathways by which microbes use nitrite and nitrate as electron acceptors for anaerobic respiration (see Figure 1). The first one is ***denitrification***, a multi-step process by which nitrate is reduced to N_2O (fungal) or all the way to N_2 (bacterial denitrification).[16] This process de facto eliminates nitrogen from the soil, by turning it into gaseous products. Finding ways to downregulate or circumvent denitrification is therefore a possible strategy to reduce overall fertilizer use in

agriculture, which we believe is an approach that deserves more attention in the field. In particular, scientists need to determine the major microbial source of N_2O that is released into the atmosphere, and how this depends on environmental conditions.

In the first chemical step of denitrification, nitrate is reduced by two electrons to nitrite by Mo-dependent nitrate reductases (NORs) via an O-atom transfer. Nitrite is then reduced to NO by either Cu- or heme-containing nitrite reductases (CuNIR and heme cd_1 NIR) in a one-electron process.[17] Interestingly, microbes never have both of these types of NIRs. Although cd_1 NIR is more abundant than CuNIR (about three-quarters are cd_1 NIRs), CuNIR is found in a wider range of ecological niches including geothermally heated environments. The three classes of CuNIRs are differentiated by the number of subunits contained within the enzyme.[19] In general, a Cu Type I center accepts an electron from an external donor, and transfers it to a Cu Type II center (with His_3 coordination) that serves as the active site and catalyzes nitrite reduction. Heme cd_1 NIRs are homodimers, and contain an unusual heme d_1 cofactor in the active site.[18] The exact role of the unusual heme d_1 for catalysis is still awaiting clarification. Although the overall mechanism of nitrite reduction by these enzymes is well understood, the details of the reaction, especially with respect to second coordination sphere (SCS) effects, are currently under active investigation. In our opinion, the role of the SCS for nitrite reduction in these enzymes needs further clarification. Generally speaking, proton transfer (assisted by the SCS) to nitrite, coordinated to the reduced cofactor, induces the generation of NO and one equivalent of water, but the details of these processes are ill defined. CuNIRs in particular are a perfect platform to further investigate how electron transfer to the catalytic center can be coupled to long-range proton transfers, which has implications for proton-coupled electron transfer (PCET) processes in many other enzymes as well.[20] Molecular dynamics and QM/MM studies have great potential to provide further, molecular-level insight into these processes.

The reduction of NO by NO Reductases (NORs) is the key step in denitrification responsible for the large scale production of the environmental pollutant N_2O, and therefore, of high significance.[1, 21] In fungi, this is the last step in denitrification, and NO reduction is catalyzed by a Cyt. P450-type enzyme, Cyt. P450nor.[18] Catalysis starts off with NO binding to the ferric heme-thiolate active site of the enzyme. The Fe(II)-NO^+ type electronic structure of this species enables direct hydride transfer from NAD(P)H to the coordinated, electrophilic nitrosonium ligand, generating an Fe(II)-NHO type intermediate (or the corresponding, protonated Fe(III)-NHOH•/Fe(IV)-$NHOH^-$ species),

which has been trapped and which is reactive towards the second molecule of NO. Unfortunately, the exact nature of this intermediate and how the reaction proceeds from there are not known (other than from computational predictions). Going forward, more research needs to be conducted on the second part of the catalytic cycle of Cyt. P450nor, to elucidate the important N-N coupling step on the road to N_2O formation. For bacterial NORs (NorBC or cNOR), on the other hand, mechanistic information is sparse. This enzyme is considered an evolutionary ancestor of heme-copper oxidases, and contains a bimetallic heme/non-heme iron active site.[18] However, whether this enzyme functions via a key mono- or dinitrosyl intermediate, responsible for N-N coupling, is unknown (other than from computational studies) and in fact, even the sequence of NO binding to the active site of this enzyme is still under active investigation.[22] Another related question is whether a NorBC analog could catalyze NO dismutation, i.e. conversion of 2 NO into N_2 and O_2. Direct evidence for this process, however, is still missing.[21] As we see it, the complex chemistry of NORs, especially the steps following N-N coupling, are another area of great need for further investigation in the N cycle.

The final step of bacterial denitrification is catalyzed by Nitrous Oxide Reductase (N2OR), which is a copper enzyme with an interesting Cu_4S cluster (called "Cu_Z") in the active site.[1, 21] Although some mechanistic insight about this enzyme has been obtained, key questions, including the structure of Cu_Z in the catalytically active form of N2OR and the binding mode of substrate to the cluster, have remained controversial. Since this enzyme reduces N_2O to N_2, it could potentially help reduce N_2O release from agricultural soils that originate from bacterial denitrification. A key question here is what substrate or cellular resource is limiting full bacterial denitrification to N_2, and whether there is a way to stimulate N2OR.

The second pathway that allows microbes to use nitrite and nitrate as electron acceptors for anaerobic respiration is ***dissimilatory nitrate reduction to ammonia (DNRA)***. In our opinion, this pathway deserves way more attention from the scientific community, since it constitutes a natural mechanism to convert nitrite and nitrate back into ammonia (i.e., fertilizer), instead of converting it into gaseous products. Stimulation of this pathway could therefore not only remediate pollution, but also reduce the total amount of fertilizer needed for crop growth. The key step in the DNRA pathway is the six-electron reduction of nitrite to ammonia, catalyzed by multiheme Cyt *c* nitrite reductases (C*c*NIRs).[17, 23] This reaction is highly complex, and mechanistic insight into nitrite reduction and relevant intermediates is hampered by the total of five hemes

present per *Cc*NIR monomer, and the fast turnover frequency of these enzymes. The active site heme is unusual, as it shows axial Lys coordination, although the significance of this Lys for catalysis is unclear.

In summary, to help mitigate the environmental problems related to N_2O emission from agriculture, we clearly need a better understanding of both the denitrification and the DNRA pathways, starting with the diversity and physiology of the microbes involved. For example, our knowledge about denitrification is largely based on detailed studies of relatively few organisms. However, given the impressive microbial diversity in Nature, we expect that there are differences in how other organisms reduce nitrogen compounds, in terms of the enzymes involved, efficiencies, and the amount of N_2O lost. In addition, we need to know more about the factors that regulate these pathways, including enzyme assembly and maturation processes, and how the DNRA vs the denitrification pathway can be stimulated. Finally, we need to improve our knowledge about the key enzymatic reaction mechanisms, especially related to the key N-N coupling and N-O cleavage steps in NORs, the activation of N_2O by N2ORs, and the mechanism of *Cc*NIRs. This knowledge will enable us to ultimately promote nitrate reduction via the DNRA pathway while simultaneously limiting nitrous oxide release during denitrification.

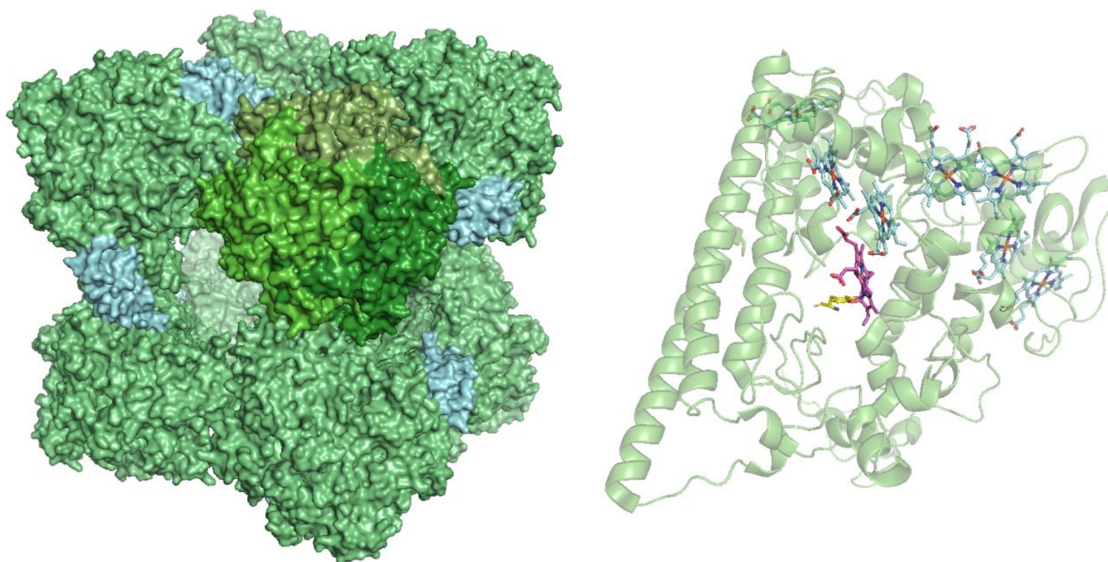


Figure 3. (left) Quaternary structure of *Kuenenia stuttgartiensis* hydrazine dehydrogenase (HDH) determined via X-ray crystallography. The *α* subunits are shown in green, with individual components of one of the eight homotrimers shown in different shades of green. The *β* subunits are shown in cyan. (right) X-ray crystal structure of the *α* subunit of HDH with the eight *c*-type hemes. The 7 electron-transfer hemes are shown in cyan while the P460-heme is shown in magneta and the Tyr462 crosslink is shown in yellow.

Anaerobic ammonium-oxidizing (ANAMMOX) bacteria are the only microbes that use hydrazine (N_2H_4) as a primary metabolite. The complexity of the ANAMMOX pathway and the enzymes involved surpasses even the most complex enzyme systems discussed so far, and therefore, progress in our understanding of the mechanisms of the involved enzymes and the chemistry that they mediate has been slow.[21] In the first step of ANAMMOX metabolism, Hydrazine Synthase (HZS), a multiheme enzyme with a complex structure (a dimer of heterotrimers), catalyzes the generation of hydrazine from NO (which originates from nitrite reduction) and ammonia via two half reactions. Hence, this is a second pathway, besides nitrification, that competes with plants for ammonia fertilizer. First, one equivalent of NO is reduced to hydroxylamine at the first active site of HZS, and then diffuses through the enzyme complex to the second active site, where it is combined with ammonia to generate hydrazine. However, in our opinion, there are still many uncertainties about this mechanistic proposal, and more work is needed to determine how these two reactions are orchestrated to ultimately form hydrazine. It is also not clear how the reaction of two nucleophiles, hydroxylamine and ammonia, can be facilitated by a heme to form an N-N bond between them. To us, this indicates that hydroxylamine might not be the correct intermediate of this process, but this remains to be determined. In the second step of the ANAMMOX process, hydrazine is then oxidized to N_2 by Hydrazine Dehydrogenase (HDH). Figure 3 shows the 3D crystal structure of the massive 1.7 MDa multiheme protein complex of HDH, which is roughly cube-shaped and comprises two subunits, α and β, in an $(\alpha_3)_8\beta_{12}$ arrangement, with a total of 192 hemes. Interestingly, the α subunits are octaheme proteins like the subunits of HAO and utilize a P460-type heme for hydrazine oxidation. Via this process, HDH returns an estimated 50% of the total N_2 to the atmosphere, to close the N cycle.[21] However, the mechanistic details of all of those fascinating reactions in the ANAMMOX pathway have remained mysterious.

Conclusions.

In conclusion, the N cycle is full of complex chemistry, unusual enzymes and multimer architectures, multi-element active sites, and useful reactions – chemistry that fascinates and perplexes at the same time. Yes, a lot remains to be learned about the N cycle, from microbial ecology to enzyme mechanisms (as pointed out above), and the stakes are high: both from an environmental point of view, but also with respect to technologically

useful and important reactions. The chemistry of the N cycle clearly deserves more attention, and we are looking forward to exciting future discoveries in this area.

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