A child-centric microbiology education framework

Evolution of nitrogenase:
Providing Nutrients to Feed a Hungry World

Dad, how are we able to produce enough food to feed so many people?

A variety of cereal grains and their products (bread, porridge, wheat) sustain our global human population. Cereal grains like those shown in the image provide nearly 80% of the protein and 50% of the calories consumed by humans and livestock across the world. Identifying, understanding, and overcoming the limitations on food production could alleviate malnutrition and hunger. Image credit: Peggy Greb, United States Department of Agriculture (USDA)-Agriculture Research Service.

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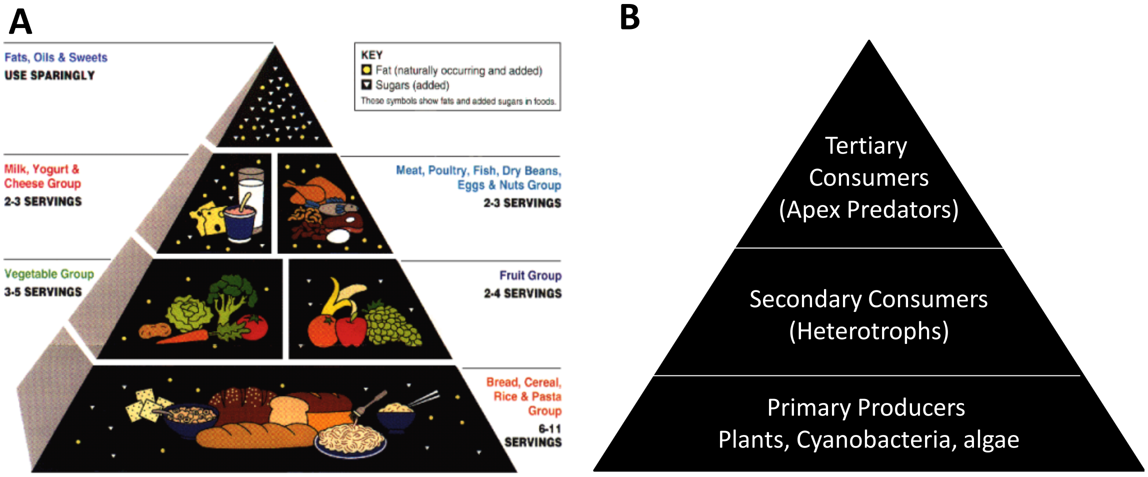
Storyline

All living organisms, at a minimum, require 1) energy to perform work and 2) nutrients to build and maintain cellular components, including proteins, lipids, and nucleic acids. This is true not only for organisms that we are familiar with, such as plants and animals (domain Eukarya), it also true for less familiar single-celled organisms that belong to the domains Bacteria and Archaea. Humans obtain energy to perform work and use nutrients to build and maintain their bodies (our bodies are made of cells) through the food that we eat, although in some cases we supplement our diets with vitamins or other compounds to help us meet our nutritional needs when our diets are insufficient. Nearly all the food that humans eat ultimately derives from plants. This includes corn, wheat, rice, beans, oats, and other grains. It is interesting to note that this also includes the meat, eggs, fish, and other food sources that we may eat, since production of these food sources requires the consumption of plants and/or their biomass. For example, chickens eat plant grains and produce eggs from this grain. Likewise, many fish eat invertebrates that are sustained by eating algae or Cyanobacteria, the latter of which can be considered microbial plants. This is called a trophic structure, or the partitioning of biomass among different organism components of an ecosystem.

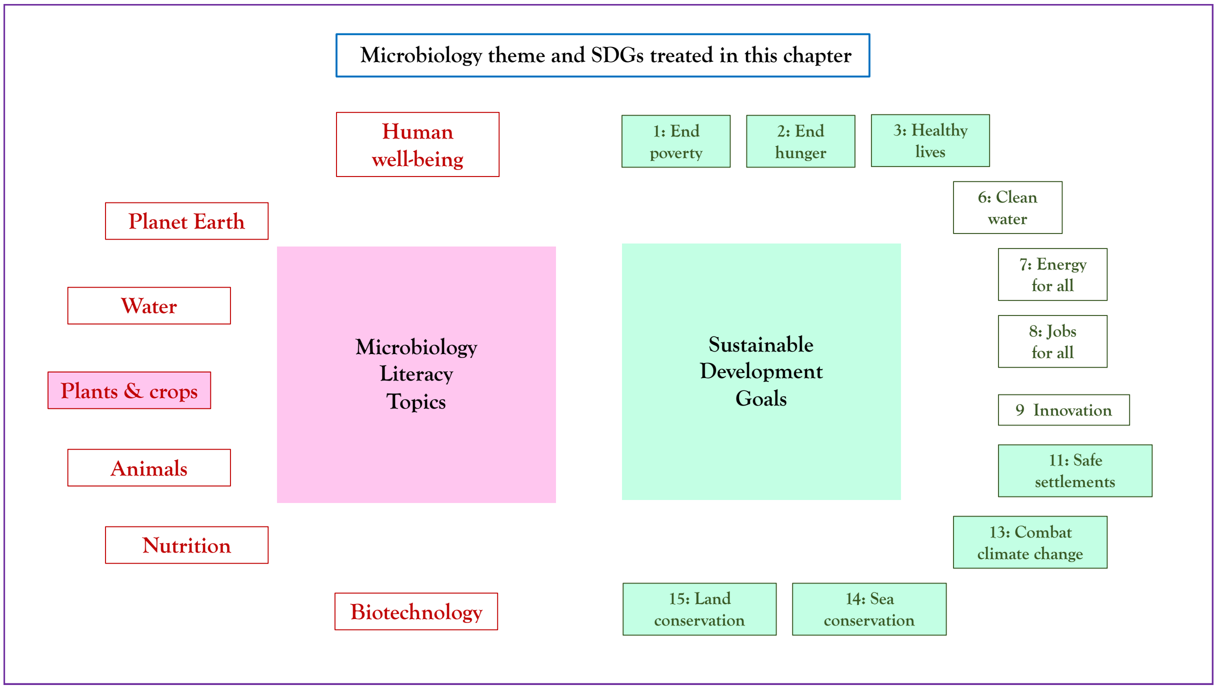
Plants, algae, and Cyanobacteria obtain energy to grow by absorbing solar radiation (light). They use this solar energy, along with water, to convert carbon dioxide (CO_2) gas from the atmosphere into organic carbon in the form of carbohydrates, or sugars, lipids, and proteins through a process known as photosynthesis. Interestingly, photosynthetic organisms obtain most of the other nutrients that they need to build and maintain cellular components from their local environment (e.g., soils, in the case of plants). However, many of the nutrients that these organisms obtain from their local environment are made available by Bacteria and Archaea. In other words, photosynthetic organisms are dependent on the activities of Bacteria and Archaea to make nutrients available to them. This is particularly true of plants. Since humans (and other animals) are dependent on plants as sources of energy and nutrients, we too are ultimately dependent on the activities of Bacteria and Archaea to provide critical nutrients. One key nutrient that all life requires is nitrogen (N). The most abundant form of N on Earth is dinitrogen (N_2) gas that makes up 79% of our atmosphere. However, this abundant form of N is not bioavailable to plants, animals, or humans, meaning they cannot uptake N as a nutrient when it is in the form of N_2. Arguably, one of the most important enzymes to evolve on Earth is nitrogenase. Nitrogenase converts N_2 to bioavailable forms of N that sustain all forms of life on Earth.



A. The food pyramid depicts the servings of various types of foods that are recommended for daily consumption by humans. Foods at the base of the pyramid (bread, cereal, rice, pasta, vegetables, and fruits) are recommended to be consumed in greater proportions than those at the top of the pyramid (milk, yogurt, cheese, meat, fish, dry beans, eggs, and nuts). Fats, oils, and sweets should be eaten sparingly. Note that foods at the base of the pyramid are plants or plant biomass (grains) whereas those at the top of the pyramid tend to be from animal sources. One can also consider the food pyramid to represent the trophic structure of natural ecosystems whereby plants, supported by photosynthesis, provide energy and nutrients to support higher trophic levels comprising animals, including humans. Image credit: USDA. **B.** A simplified trophic structure of photosynthetically supported ecosystems shows the base of the pyramid comprises primary producers whose biomass sustains a slightly smaller amount of biomass (smaller portion of the pyramid) in the form of secondary consumers (e.g., cows, pigs, deer) that themselves can support a smaller amount of biomass of tertiary consumers or apex predators (e.g., lions, humans, bears). Note that tertiary consumers can also be omnivores, or organisms that consume biomass of primary producers and secondary consumers. Humans are an example of omnivorous organisms, whereby an individual might eat bread (made of plant grains) or eggs produced by chickens (secondary consumers of plant grains).

The Microbiology and Societal Context

The microbiology: trophic groups; food webs and trophic levels; nitrogen; evolution; nitrogenase; metal cofactors of enzymes; enzyme oxygen sensitivity; eutrophication. *And, peripherally for completeness of the storyline*: Haber-Bosch process for nitrogen reduction. *Sustainability issues:* poverty; hunger; health; sustainable cities; climate action; water and soil conservation.



Nitrogenase: The Enzyme and Its Microbial Hosts

1. ***Nutrition***. Before we begin discussing the role of the microbial enzyme nitrogenase in sustaining global nutrition, let's take a step back and revisit what makes up a cell. Cells comprise proteins, lipids, and nucleic acids, among other components. These biomolecules largely are made of the elements carbon (C), oxygen (O), hydrogen (H), nitrogen (N), and phosphorus (P). Organisms obtain most of their O and H by assimilating water (H_2O), one of the many reasons that plants and animals require this compound. However, it is the other elements that comprise biomass (C, N, and P) that are our primary focus here. On average, cell material has a C to N to P ratio (C:N:P) of 106:16:1. This means that for every atom of P that a cell needs, it needs 16 atoms of N and 106 atoms of C.

When thinking about where a cell obtains these elements, it is useful to divide life forms into two groups: autotrophs and heterotrophs. These two groups are distinguished by where they obtain the C necessary to construct cellular material. Autotrophic organisms obtain their C through photosynthesis, whereas heterotrophic organisms obtain their C by consuming C produced by autotrophs. There are also autotrophic organisms that are not dependent on light, termed chemoautotrophs, but since they only contribute about <1% of the organic C produced on Earth today, they will not be discussed further in this article. Cyanobacteria (domain Bacteria), algae (domain Eukarya), and plants (domain Eukarya) are all autotrophs. Heterotrophs, including animals, invertebrates, and humans, are common among members of the Bacteria, Archaea, and Eukarya and are all dependent on autotrophs. For this reason, autotrophs are often considered keystone species, or species whose activities have a disproportionate effect on the functioning of an ecosystem.

Above, we discussed differences in where autotrophs and heterotrophs obtain their C. Now let's discuss the general ways that these groups of organisms obtain N, arguably the element whose availability most commonly limits production of plants, Cyanobacteria, and algae. All forms of life have a very high demand for N since it is a component of amino acids (the building blocks of protein) and nucleic acids (the building blocks of DNA and RNA), that are two key components of cells.

The most abundant source of N on the planet is in the form of N_2 gas which, as mentioned above, comprises 79% of the air that we breathe. Yet, N_2 is chemically inert and is not bioavailable to life. Rather, it first must be converted (or fixed) to a form of N that biology can use. Such forms include the most oxidized form of N, nitrate (NO_3^-), and the most reduced form of N, ammonia (NH_3). In the case of NO_3^-, cells must first chemically reduce this compound to NH_3 before it can be assimilated and used to meet N demands. However, the biochemical reaction to reduce NO_3^- to NH_3 requires energy and, for this reason, NH_3 is the preferred form of N for Bacteria, Archaea, and Eukarya.

Animals and humans obtain fixed N from the foods that we eat, in particular those rich in protein. In essence, we eat food that contains amino acids and nucleic acids and digestion of these compounds in our gastrointestinal tracts releases the NH_3 that is then absorbed by our cells. Since all nutrition for animals and plants ultimately comes from autotrophic organisms, our focus remains on how autotrophs obtain NH_3. So, how do such organisms obtain N from the environment when the most widely available source of N (N_2) is not available to them?

2. ***Bioavailability of nitrogen on early Earth and the origin of nitrogenase.*** Microbial life emerged on Earth ~3.8 billion years ago (Gya). Yet, it was not until ~2.8 Gya that oxygenic (oxygen-producing) photosynthesis evolved and an additional 2 billion years (Ga) before land plants evolved (~800 million years ago (Mya)). As mentioned above, on Earth today non-photosynthetic-based life (termed chemosynthetic life) accounts for ~<1% of the organic carbon production (primary production) on the planet. Between 3.8 and 2.8 Gya, it is reasonable to assume that all of Earth's ecosystems were similarly or slightly less productive as non-photosynthetic ecosystems are today, representing about ~<1% of today's global productivity. In essence, without oxygenic photosynthetic organisms like Cyanobacteria, algae, and plants, the Earth could only support a small fraction of the life that it does today. Yet, the microbial life that existed on Earth between 3.8 and 2.8 Gya would have still needed a source of bioavailable N (i.e., NO_3^- or NH_3) to support biosynthesis of protein and nucleic acids. What was the source of bioavailable N for microbial life during this time?

Scientists have long known that lightning, which forms due to discharge of electrostatic energy in Earth's atmosphere, can break the triple bond between N atoms (N≡N) in N_2 allowing them to recombine with O in the atmosphere to form oxidized N compounds, such as NO_3^-. Early Earth was volcanically more active than Earth today, and volcanoes can spark lightning storms, suggesting that some NO_3^- might have been produced by lightning that could have supported microbial N demands at this time. Alternatively, reduced minerals in Earth's mantle and crust, when exposed to N_2 at high temperatures, can promote the reduction of N_2 to form NH_3. Generally, scientists believe that these processes provided bioavailable N to early forms of life on Earth.

Lightning strikes near Lake Wright Patman, Red River, Texas, U.S.A. Discharge of electrostatic energy in the form of lightning can beak the triple bond (N≡N) in N_2, allowing N atoms to recombine with O atoms in the Earth's atmosphere to create oxidized N compounds that are bioavailable. Image credit: Griffinstorm and is used here under the Creative Commons Attribution-Share Alike 4.0 International license.

However, the supply of bioavailable N from these sources would have been finite and it is easy to imagine that microbial life maximally exploited this supply, driving N to be the nutrient that was limiting to the biosphere. Then, 2.8 Gya, oxygenic photosynthetic organisms evolved, making expansion of the biosphere possible (eventually by 2 orders of magnitude) by using a seemingly endless energy supply (i.e., the sun) to form organic C. However, the fierce competition for bioavailable N imposed limitations on such rapid proliferation. What was the microbial biosphere to do?

The limitation of bioavailable N must have imposed an enormous pressure on biology to evolve a microbial solution to "tap" the vast N_2 reserves present in the atmosphere. Roughly 2.1 Gya, through many generations of mutation and selection, ultimately an enzyme that would forever change the planet emerged – *nitrogenase*. Finally, one of the strongest chemical bonds known on Earth, the stable N≡N triple bond of N_2, had met its biological match. The nitrogenase enzyme catalyzes the chemical reaction that reduces the triple bond (N≡N) of N_2 to NH_3 making N from the atmosphere bioavailable. With nitrogenase having evolved, biology had what seemed to be a near endless supply of bioavailable N, allowing photosynthesis-supported ecosystems to proliferate.

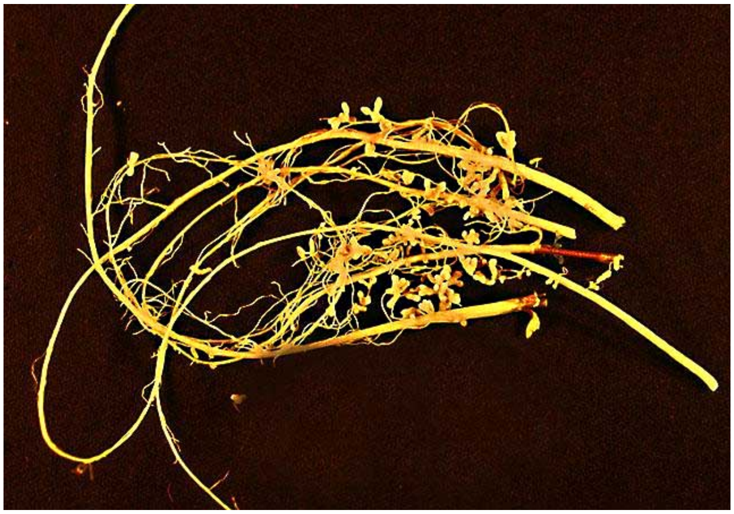
3. ***How did nitrogenase evolve?*** Evolution tends to act on existing variants in a population. In this case, evolution acted on an enzyme that is involved in reducing a double bond in a co-factor (termed co-factor F_{430}) of critical importance to methanogenesis, a microbially-mediated biological process that generates methane (CH_4) as a product and that is one of the most ancient metabolic processes that arise in single-celled organisms. Through multiple iterations of mutation and selection on variations of co-factor F_{430}, the enzyme nitrogenase originated.

It is thought that the ancestral methanogenic microorganism where nitrogenase first originated was an extremophile, or an organism that lives under conditions that are very different than those suitable for human life. Specifically, available evidence indicates that this ancestral methanogen combined hydrogen (H_2) and carbon dioxide (CO_2) as a source of energy (generating CH_4 and H_2O as products) and inhabited a high-temperature and high-pressure environment, conditions that lower the amount of energy needed to reduce N_2 to NH_3. This will become important later when we discuss the Haber-Bosch process of industrial N_2 reduction to NH_3.

Methanogens are anaerobic organisms, meaning that they cannot grow and proliferate in environments where oxygen is present. As such, many of the enzymes that methanogens use to support their metabolism, including nitrogenase, are highly sensitive to oxygen. While available data indicate that nitrogenase first originated in an anaerobic methanogen and in an environment that was oxygen free, it was not long until aerobic Bacteria acquired nitrogenase through a process called lateral gene transfer, where genes are shared among distantly related organisms. After all, oxygenic photosynthesis had already originated by 2.1 Gya (when nitrogenase is thought to have evolved) and O_2 was starting to accumulate on Earth, capable of supporting organisms that respire O_2 (aerobes). Once aerobic Bacteria acquired the genes that encode for nitrogenase enzymes, they had the capability to reduce N_2. All would have been excellent for these aerobic Bacteria if not for the fact that nitrogenase is sensitive to O_2. So how can an O_2-sensitive enzyme function in an aerobic bacterium that inhabits environments rich in O_2?

Nitrogen-fixing Bacteria have evolved several unique ways in which they can protect O_2-sensitive nitrogenase from being inactivated by O_2. Perhaps the simplest of these mechanisms is for aerobic Bacteria to separate nitrogenase either spatially or temporally from O_2-dependent processes. For example, some Cyanobacteria, organisms that make O_2 during oxygenic photosynthesis during the day, only use nitrogenase at night. During the night, oxygenic photosynthesis is inactive (phototrophs need solar radiation to drive oxygenic photosynthesis) and any O_2 in the environment is quickly consumed by neighboring aerobic microorganisms rendering the environment O_2-free and allowing nitrogenase to function. Still, other Cyanobacteria create differentiated cells called heterocysts. These cells are not capable of oxygenic photosynthesis and thus do not generate O_2. These cells are exclusively made to house nitrogenase in an otherwise O_2-free environment and are cross-fed organic carbon from non-heterocyst cyanobacterial cells that are conducting oxygenic photosynthesis. In turn, heterocysts transfer fixed N to non-heterocyst cells to support photosynthesis. Finally, some cells secrete a thick polysaccharide sheath (mucus layer) outside of the cell to minimize O_2 diffusion into the cell. These same cells increase the rate that they use O_2 so that the cytoplasm remains O_2 free, allowing nitrogenase to function.

These critical physiological strategies permit N_2 fixation to occur at levels needed to support Earth's fixed N demands. Today, nitrogenase is found in many groups of Bacteria and Archaea, aerobes and anaerobes, and autotrophs and heterotrophs, including well-known symbionts of important crop plants, like root nodule-forming rhizobia that provide nitrogen to soybean. The extent to which microbes have evolved strategies to maintain nitrogenase function in the presence of O_2, combined with the diversity of organisms and metabolic backgrounds where nitrogen fixation functions, points to the fundamental role that this enzyme has in supporting life on Earth. So, what exactly is nitrogenase and how does it reduce N_2 to NH_3?



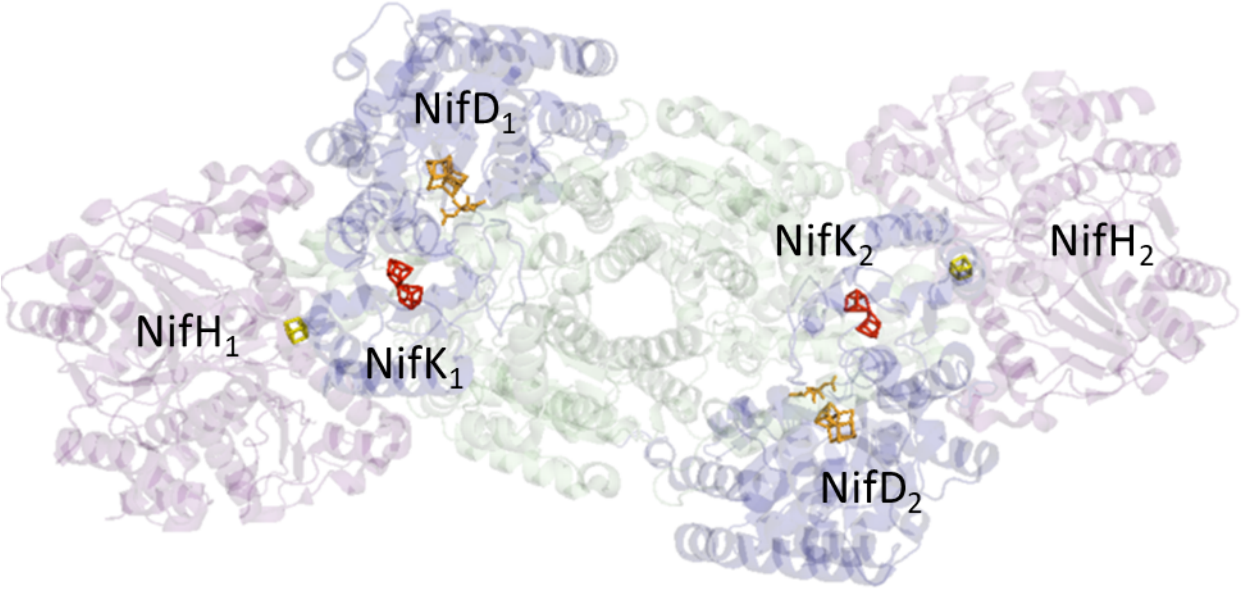
The roots of a soybean plant are colonized by members of the bacterial genus, *Rhizobia*. The soybean creates nodules that house the *Rhizobia* cells that provide fixed N to the plant through the activity of nitrogenase. In return, the plant provides the *Rhizobia* with organic carbon to fuel its metabolism and to drive reduction of N_2. Such partnerships, or symbioses, are common among agriculture crops. Are there ways that we can stimulate the occurrence and extent of such symbioses in nature to improve crop production? Image credit: United States Department of Agriculture.

4. ***Nitrogenase biochemistry.*** Nitrogenase is one of the best studied enzymes, due in no small part to the critical role that it has in providing bioavailable N to support life on Earth. That said, the biochemistry of nitrogenase, while fascinating, is also extremely complex. Many of these complex details are unnecessary for the purposes of this article – to introduce the enzyme and its ecological, economic, and agricultural importance. Thus, our discussion of the biochemistry of nitrogenase will be kept to a minimum in this article.

Enzymes are nanometer-sized molecular machines/catalysts that make life on Earth possible by accelerating the rates of chemical reactions. Often, the way that enzymes speed up chemical reactions is by incorporating key catalytic metals and other elements into what are known as active sites - the place in an enzyme where the chemical transformation takes place.

There are three forms of nitrogenase found in modern day bacterial and archaeal cells (nitrogenase does not occur among Eukarya) that are differentiated by the metals found in the active site. The active site co-factor of the most common form of nitrogenase contains the elements molybdenum (Mo), iron (Fe), sulfur (S), and C. It is at the Mo atom where the action takes place and N_2 is reduced to form NH_3. The reduction of N_2 is energy intensive, requiring 16 molecules of adenosine triphosphate (ATP; the energy currency of all cells) and 8 electrons (e^-) to reduce a single molecule of N_2 to 2 molecules of NH_3. Other forms of nitrogenase have a vanadium (V) or Fe atom in place of Mo in their active site; these enzymes are less efficient than Mo-dependent nitrogenase. All of these enzymes are evolutionarily related and the V- and Fe-dependent forms typically only function when cells are limited for Mo and cannot synthesize the active site of the Mo-dependent enzyme; they serve as back-ups for the crucial process of nitrogen fixation. Further, the V- and Fe-dependent forms evolved after Mo-dependent nitrogenase and the Mo-dependent nitrogenase is responsible for the majority of biological N_2 reduction on Earth today. As such, we will focus our discussion only on Mo-dependent nitrogenase.

Mo-dependent nitrogenase, also called Nif, comprises three individual proteins (called subunits) termed NifH, NifD, and NifK. NifD and NifK associate with each other and house the catalytic active site; these are commonly referred to as the "structural proteins" of nitrogenase. NifH, also called the "iron protein" of nitrogenase, also associates with NifDK, but only temporarily. Each time NifH "docks" with NifDK, e^- are delivered and used to reduce N_2. Each Mo-dependent nitrogenase comprises two sets of NifHDK subsunits ($NifH_{1,2}D_{1,2}K_{1,2}$) and one set is a mirror image of the other set. As such, Nif is a rather large enzyme that has a long path for electrons to travel from the electron donor protein subunit (NifH) to the active site that is housed in NifDK, where the electron acceptor (N_2) is reduced.



The three-dimensional structure of the enzyme, Mo-nitrogenase (or Nif). Depicted is the heterotetramer NifDK with a NifH docked on each side. Each subunit of the enzyme, $NifH_1$, $NifH_2$, $NifD_1$, $NifD_2$, $NifK_1$, $NifK_2$) are shown. Note that if you divide the image in half, one side is a mirror image of the other. Electrons are delivered through a series of iron-sulfur molecules ultimately to arrive at the active site of the enzyme (two series of electron delivery paths are shown, one for each NifHDK unit). The first iron sulfur molecule is depicted in yellow in each half, the second is depicted in red, and the third active site iron sulfur molecule is depicted in orange. Consider these to behave like a wire connecting a light switch to a lightbulb. NifH is the switch, the iron-sulfur molecules are the wire, and N_2 is the lightbulb. It is amazing to think that this tiny, nanometer-sized molecular machine could have such a monumental effect on the evolution and production of life on Earth. Image credit: Mlee17 and is used under the terms of the GNU Free Documentation License, Version 1.2.

A series of conductive iron-sulfur atoms line a pathway to transfer or "wire" electrons to the active site where N_2 reduction takes place. A chemical reaction, called hydrolysis, of ATP changes the conformation (shape), of NifH that enables association/dissociation from NifDK, allowing an e^- to be delivered. As mentioned above, the reduction of a single molecule of N_2 to 2 molecules of NH_3 requires 8 e^-; the 16 molecules of ATP (forming adenosine diphosphate (ADP) and inorganic phosphate (P_i) as products) allow for multiple cycles of electron delivery to nitrogenase (i.e., association/de-association of NifH from NifDK). While the overall reaction of N_2 fixation by Mo-dependent nitrogenase consumes 8 e^-, the reduction of N_2 to NH_3 only requires 6 e^-. Where do the other 2 e^- go? It turns out that H_2 is also a product of N_2 reduction – reduction of protons (H^+) to a H_2 molecule accounts for the other 2 e^-. In total, the reduction of N_2 to NH_3 occurs according to the equation below:

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

5. ***Evolutionary significance of nitrogenase.*** It is safe to say that the evolution of nitrogenase some 2 Gya put the biosphere on a path to support higher forms of life, including plants, animals, and humans. Without nitrogenase having evolved, it is difficult to see how such forms of life (including each of us) would have evolved. Today, nitrogenase is responsible for generating approximately half of the bioavailable N on the planet and can be argued to support half of all life on the planet. This natural fertilizer is what supports much of the food production capacity of our agricultural system and is what supports the natural function of Earth's ecosystems that maintains clean air, water, and ecosystem health. But where does the other half of the bioavailable N come from to support the N demands of Earth's biosphere?

6. *Haber-Bosch Process: industrial reduction of nitrogen and its societal impacts*

a. **Overview.** The industrial process known as the Haber-Bosch process of N_2 reduction generates the remaining half of fixed N on Earth. The Haber-Bosch process is a synthetic way of reducing N_2 to form NH_3 and is where the N in fertilizers used in our lawns and our agriculture fields originates. The Haber-Bosch process was invented in the 1940s and is carried out at high temperature and high-pressure using metal (typically iron powder) catalysts and H_2 as a source of electrons.

It is perhaps of little coincidence that these characteristics of how the Haber-Bosch process is carried out are strikingly similar to those inferred for the ancestral methanogen where nitrogenase first originated – in a H_2-dependent methanogen living in a high temperature, high pressure hydrothermal environment with a nitrogenase enzyme that comprises numerous iron-sulfur molecules and catalysts. It is possible that such narrow conditions represent the only suitable conditions that favor such reactions to take place on relevant time scales.

It is perhaps also worth pointing out that the Haber-Bosch process is highly sensitive to product (i.e., NH_3) accumulation and thus NH_3 that is produced by this process must be removed so that it does not build up and inhibit the forward reaction (formation of NH_3). Adding further intrigue to the biological origins of nitrogenase is that hydrothermal vents (where an H_2-dependent methanogen originating nitrogenase likely inhabited) are highly diffuse environments where products would be readily removed by mixing with adjacent ocean waters.

b. **Potential Impacts on Climate Change.** Like nitrogenase, there is no question that the Haber-Bosch process has allowed for extraordinary increases in the productivity of Earth, with some estimates suggesting that roughly half of the world's human (and thus animal) population is supported by N produced by this process. Simply put, there would be no conceivable way that we could support the food production needed to feed Earth's human population without it. Yet, there are clear downsides to our dependence on the Haber-Bosch process. In particular, the H_2 that is used to reduce N_2 in the Haber-Bosch process is derived from CH_4 that originates from fossil fuels such as natural gas, coal, or petroleum. Further, fossil fuels are used to create the high temperature (>400°C) and pressure (>10 MPa) conditions that are required to efficiently operate the catalyst for the Haber-Bosch reaction. It is estimated that >3% of the world's carbon emissions from burning of fossil fuels (primarily natural gas used to produce H_2) is attributed to the Haber-Bosch process.

c. **Potential Impacts on Water Quality.** It is also important to consider the fate of NH_3 that is produced by the Haber-Bosch process. Biological nitrogenase generates NH_3 within the cell and it is quickly assimilated into organic forms that are either retained within the cell or are carefully transferred to symbiotic partners, thereby minimizing waste. However, industrial sources of NH_3 are commonly applied on the surface of agricultural soils as anhydrous ammonia.

The application of NH_3 can stimulate a microbial process called nitrification that converts NH_3 into nitrate (NO_3^-). NO_3^- is easily dissolved in water, such as rain or irrigation, and can be flushed from fields and into rivers, and ultimately oceans. This pulse of NO_3^- to aquatic systems leads to excessive nutrient loading, or eutrophication, and can cause massive blooms of Cyanobacteria and algae. When these blooms of Cyanobacteria and algae die, they contribute large amounts of biomass that stimulate heterotrophic metabolism and consumption of O_2 rendering the local environment O_2-free. These conditions suffocate aerobic invertebrates and fishes forming "dead-zones".

Further, NO_3^- can infiltrate local aquifers that serve as sources of water for human populations. NO_3^- is a carcinogen and additional water treatment practices are necessary to remove this compound from water supplies prior to its consumption.

Are there alternative ways to increase biological production of NH_3 that do not have the deleterious side effects associated with industrial scale NH_3 production and fertilization? Alternatively, are there ways to enhance the retention of NH_3 applied to agricultural fields to maximize crop production by minimizing downstream ecosystem effects?

Relevance for Sustainable Development Goals and Grand Challenges

- **GOALS 1&2: End poverty and hunger**. Nitrogen fixation has a profound agronomic, economic, and ecological impact due to N availability commonly limiting agricultural production that supports the human and animal population of the world. Yet, many people suffer from the inextricably linked issues of hunger and poverty, with ~10% of the world's population being undernourished and ~9% of the world's population living in extreme poverty. Promoting best practices in agriculture, including in areas of the world that practice traditional agriculture, could be used to help alleviate both problems. Cyanobacteria and algae form crusts (mats) on soils that 1) stabilize the soil, 2) help the soil retain moisture, and 3) fertilize the soil. In the case of Cyanobacteria, many encode nitrogenase and can promote the production of bioavailable N to further fertilize the soil. Establishing no-till agriculture practices helps to keep these mats intact and can promote nutrient/water retention and soil fertility, thereby enhancing crop productivity. A more productive agricultural plot can provide more food that can be sold as a source of revenue (decrease poverty) and can feed more people (decrease hunger).

  But, are there other ways that developed countries can help end poverty and hunger in less developed countries? At first order, government, academic, and industry scientists are trying to understand the basic mechanisms that enable nitrogenase function (see biochemistry section above). The idea is that by understanding how the enzyme functions, scientists can improve it through genetic engineering approaches or use their knowledge to develop synthetic, biology-inspired catalysts to increase the economics and yield of crop production. For example, if scientists could learn how to direct energy from sunlight to nitrogenase or a nitrogenase-like catalyst (cutting out the photosynthetic apparatus), they could essentially use unlimited amounts of seawater (water as the source of e^-), unlimited amounts of light as the source of energy, and unlimited amounts of atmospheric N_2 to develop synthetic fertilizers that could be generated anywhere on the planet. Alternatively, if nitrogenase could be engineered to be insensitive to O_2, then the substantial energy that aerobic Bacteria currently direct to protecting nitrogenase from O_2 (e.g., heterocyst formation) could otherwise be directed to generating more biomass and bioavailable N to support crops. Importantly, for any of the solutions to be realized, creative minds need to be continuously recruited to join Science, Technology, Engineering, and Mathematics (STEM) disciplines, and the governments and industries of developed nations need to continue to fund basic and applied research of nitrogen fixation and train the next generation of STEM scientists.

- **GOAL 3: Promote good health and well-being.** Good health and well-being go hand in hand with a healthy diet consisting of nutritious food. With production of at least half of the food produced on this planet coming from bioavailable N contributed by nitrogenase, this enzyme obviously contributes to the health and well-being of humanity. However, not all food is created equal with regard to its nutritional content. For example, soybeans are enriched in protein and also bioavailable N, due in no small part to the association of these plants with N_2-fixing *Rhizobia* in their roots (root nodules are a common feature of leguminous plants). In fact, leguminous plants are often planted to increase the bioavailability of N in soils. In contrast, rice, oats, and barley contain less protein and diets rich in these grains may need to be supplemented with other sources of protein. Humans only make 12 if the 21 amino acids that comprise the proteins in our bodies. In other words – we depend on a diet containing adequate amounts of those other 9 amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine). Without these amino acids, health outcomes decline rapidly. We are what we eat and eating nutritious diets that contain adequate amounts of protein with essential amino acids is only possible because nitrogenase relieves N limitation in agriculture production systems.

- **GOAL 11: Sustainable cities and communities.** Fixed or bioavailable N limits world food production. A major waste stream of bioavailable N comes in the form of urine, which gets its name from the compound urea. Urea, also known as carbamide, is the form of N that is produced during protein metabolism by mammals. It contains two amide ($-NH_2$) groups attached to a central carboxyl group. When those amines are cleaved from the molecule, they become bioavailable and can be used to meet fixed N demands. Many diverse microorganisms have been isolated from sewage treatment plants that can cleave amines from urea. Yet, cities and municipalities rightfully expend significant resources to keep urea and its derivative compounds out of local water ways, due in no small part to harmful algal blooms that can develop from feeding on urea. As such, water treatment plants are designed to promote the aerobic oxidation of urea (and ammonia) to generate NO_3^- by vigorous aeration. Then, the sewage treatment operators promote denitrification, or the reduction of NO_3^- to N_2 gas. While this makes sense from the perspective of water quality, it also represents a sink for bioavailable N that could otherwise be used to overcome bioavailable N limitation in agriculture production. It should also be noted that the denitrification process is leaky, and often can contribute nitrous oxide (N_2O) that is a potent greenhouse gas. Are there alternatives to traditional sewage treatment practices?

Constructed wetlands and agriculture systems that interface with sewage effluent are two possibilities. Common among these approaches is 1) to keep bioavailable sources of N from reaching local waterways, and 2) to enhance the retention of bioavailable N in the biosphere by promoting nutrient uptake and growth of plants along with other organisms in these systems. In essence, rather than "degas" fixed N from sewage as N_2 (and as the greenhouse gas N_2O) through the combined processes of nitrification/denitrification, these practices seek to use the fixed N in sewage to grow agricultural crops that can be harvested or fed to animals. Aquaculture practices have also evolved to make use of excretions of bioavailable N from fishes by growing productive plants in the waters that those fish inhabit. Creative solutions like these must also be cognizant of public health concerns, including pathogen release and transfer. Scaling up such systems to meet the needs of larger cities and municipalities is a challenge that needs creative solutions from the next generation of STEM scientists – *you*!

- **GOAL 13: Climate action.** Earth's climate is warming due to fossil fuel burning and emissions. As mentioned above, nearly 3% of the world's CO_2 emissions are attributed to the Haber-Bosch process of industrial N_2 reduction. It is widely argued that we need to transition away from fossil fuels as sources of transportation, heat, and electricity. It could equally be argued that we need to transition away from the Haber-Bosch process to an alternative process to generate the fertilizer needed to grow the food to feed the world. Yet, 10% of the world's population does not have enough food to eat. One cannot imagine arguing for an end to this process with that many people in need of food. To effectively transition away from the Haber-Bosch process without compromising food production capacity, we need to either engineer more efficient nitrogenases, increase the capacity for biological N_2 fixation in agricultural fields, engineer synthetic N_2 reducing catalysts, or identify better methods of retaining fixed N in the biosphere following sewage or fertilizer application. Perhaps more tangible in the short term is to come up with alternative sources of H_2 as a feed stock for the Haber-Bosch process and as a source of electricity to operate this process at high temperature and pressure. Substantial effort is underway to do just that, ranging from recovery of geological H_2 from the subsurface to developing light driven H_2 production (bio)catalysts. Ironically one of the targets for making H_2 at production levels is the enzyme nitrogenase itself, given that it releases a molecule of H_2 for every molecule of N_2 that it reduces.

- **GOALS 14&15: Life below water and on land.** Industrial agriculture, where synthetic fertilizers are applied to production fields, can have negative impacts on local and downstream ecosystems, most notably through runoff and the large fish (and other aquatic animal) kills that occur due to algal or cyanobacterial blooms from excessive nutrient inputs into waters. There is another, less well studied effect of fertilizer application: It disturbs the soil ecosystem and can select against native organisms with the capability to naturally reduce N_2. This can result in a negative feedback loop where the only way that soils can support agriculture production is if it is fertilized with synthetic NH_3. One way to break this cycle is to plant pulse crops, or legumes that form associations with nitrogen fixing Bacteria, in agricultural fields to increase soil fertility and to begin to restore native soil biodiversity. Another possible way to ensure that aquatic and terrestrial biodiversity is preserved would be to engineer plants with the ability to better assimilate and store NH_3 so that it does not have the opportunity to modify soil microbial communities or to reach water ways and disrupt aquatic communities.

While new approaches and engineered plants and Bacteria are being developed, farmers continue to make astonishing advances in what is termed precision agriculture. Farmers can monitor crop production across fields with increasing spatial accuracy (hence the word “precision”). This, in turn, allows them to identify locations in a field that are low productivity, perhaps due to N limitation. Prior to the next planting season, the farmer can precisely guide fertilizer applications to deliver more to low performing areas, while minimizing application to areas where production is high. In this way, application of fertilizer is optimized, reducing waste, improving crop production and its economics, all while minimizing downstream effects to land and aquatic ecosystems.

Potential Implications for Decisions

1. **Individual**
    - **a.** Benefits and drawbacks of nitrogenase or the Haber-Bosch process to fix N_2 into bioavailable N.
    - **b.** Ethics of bioengineering plants to contain the nitrogenase enzyme.

2. **National Policies**
    - **a.** Consider different food crops and the benefits and risks of planting them as it relates to their N content and their ability to form symbioses with microbes.
    - **b.** The role of developed countries in providing food assistance to less developed nations.
    - **c.** Consider policies for genetically modified organisms, such as those bioengineered with nitrogenase, in agriculture.
    - d. Consider policies for mitigating public risk to pathogen exposure in constructed wetlands treating N-rich human sewage.

Pupil Participation

1. **Class Discussion**
    - a. Nitrogenase originated about 2.1 Gya whereas microorganisms originated about 3.8 Gya. Discuss how different the planet would have been prior to the origin of

nitrogenase. What would continents look like without trees, forests, and plants. Would there be any soil, and how might its composition differ from what we are familiar with today? How different might the atmosphere be? What kinds of foods would be available to eat on such a planet?

b. What types of environments can you envision where organisms that make nitrogenase play a major role in supporting communities, either microbial or macrobial? Are there any environment types where you might expect to find very few organisms that encode nitrogenase?
c. If humans were to solve the problem of N bioavailability limiting crop production, what do you think the next factor limiting crop production would be?
d. If the bioavailability of N has limited plant productivity for the past ~800 Mya, why do you think plants have not acquired the ability to fix their own N_2?

The Evidence Base, Further Reading, and Teaching Aids

The nitrogen cycle (for elementary school students): https://kids.frontiersin.org/articles/10.3389/frym.2019.00041

The nitrogen cycle (for high school students): https://microbiologysociety.org/why-microbiology-matters/what-is-microbiology/microbes-and-the-outdoors/nitrogen-cycle.html

Global nitrogen cycle in the 21st century: https://royalsocietypublishing.org/doi/10.1098/rstb.2013.0164

Nitrogenase biochemistry and function: https://proteopedia.org/wiki/index.php/Nitrogenase

Glossary

ATP – Adenosine triphosphate (ATP) is an energy rich compound that all forms of life depend on to power chemical reactions and to perform work.
Autotroph – An organism that converts inorganic CO_2 to organic carbon. These organisms can either use light energy to facilitate this process (photosynthesis) or chemical energy to facilitate this process (chemosynthesis). Autotrophs often encode the enzyme nitrogenase which allows them to meet their fixed N and C nutritional demands using atmospheric N_2 and CO_2. This is quite the advantage for such organisms and is one reason that they are so productive and dominate most ecosystems.
Bioavailable – a term used to describe whether a compound can be absorbed by an organism to meet nutritional requirements. For example, dinitrogen (N_2) is not available to humans as a source of nitrogen (N). However, N_2 is available to microorganisms with nitrogenase since they can convert this to ammonia (NH_3) that can be assimilated. A second example is cellulose from plants. Humans cannot metabolize cellulose as an energy and carbon source. However, microorganisms can metabolize cellulose as an energy and carbon source. In this case, cellulose is not bioavailable to humans but is to some microorganisms.

Biomass – The components of living organisms including their cells and their cellular contents. In plants, this includes sugars (carbohydrates, cellulose) and lignin that secondary consumers depend on for growth.

Cereal Grains – The seeds produced by plants that can be harvested for human or animal consumption. Examples include wheat, rice, soybeans, and corn.

Crust – The upper most portion of the Earth that covers ~40% of its surface as continents and oceanic shelves. The crust extends to depth of up to 80 km on continents. The crust has iron-rich minerals that can react with N_2 gas to abiotically (in the absence of biology) to produce NH_3.

Dinitrogen – A compound with the chemical formula of N_2, or $N \equiv N$. The triple bond between the two N atoms makes this compound stable, which is why it comprises 79% of our atmosphere.

Food Pyramid – The food pyramid is a diagram used to illustrate the recommended amounts of food to be eaten by humans.

Heterotroph – An organism that utilizes organic carbon to meet their C and energy demands. Some heterotrophs encode the enzyme nitrogenase, which allows them to meet their fixed N nutritional demands using atmospheric N_2.

Keystone Species – An organism whose activities have a disproportionate effect on ecosystems. Autotrophs and organisms that fix N_2 are often considered to be keystone species, since removing such organisms (and their functions) from an ecosystem can cause extreme changes to that ecosystems structure and composition.

Lipids – A class of non-water-soluble biomolecules that includes fats, oils, and steroids and compose cell membranes. Lipids are composed of phosphorus, carbon, and some nitrogen.

Mantle – The inner part of the Earth that extends from the dense core up to the crust. The mantle is rich in iron containing minerals that are highly reduced (electron rich or non-oxidized) that can react with N_2 gas to abiotically (in the absence of biology) to produce NH_3.

Mutation – The change of a base in a molecule of DNA that occurs by chance when the genome of an organism is replicated. Since an organism's genome (made of DNA) contains genes that encode proteins (enzymes) that do the important work for a cell, a change in the DNA template that encodes those proteins can affect protein function in either a beneficial or detrimental way.

Nitrogenase – A complex enzyme that uses ATP and e^- to reduce N_2 to NH_3.

Nucleic Acids – Biomolecules that comprise deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and are composed of long chains of individual nucleotides, some of which are nitrogen-rich.

Omnivores – An organism that obtains nutrients and energy from both primary producers (plant biomass) and secondary consumers (animal biomass).

Photosynthesis – The conversion of inorganic CO_2 to organic carbon in the form of carbohydrates, protein, and lipid using light energy.

Precision Agriculture – A new approach to agriculture that incorporates information technology such as location specific crop yields to gauge the levels of fertilizer that is needed to be applied to that field location to optimize crop production and ensure sustainability and to minimize environmental impacts. An example of this would be to decrease fertilizer application in productive areas of a crop field and to increase fertilizer application in less productive areas.

Primary Production – A term used to describe the storage of chemical energy in the form of organic carbon. Most primary production is performed by autotrophic organisms, of which there are those dependent on light energy (photosynthetic organisms) and chemical energy (chemosynthetic organisms).

Protein – A nitrogen-rich biomolecule composed of amino acids. Some proteins are enzymes that catalyze (speed up) biochemical reactions.

Pulse Crops – Plants, from the legume family, that are harvested for their dried seeds. Examples of pulse crops include chickpeas, common beans (pinto, black, and kidney beans), dry peas, lentils, and cowpeas. Many pulse crops form root symbioses with N_2 fixing bacteria (e.g., *Rhizobia*), resulting in grains that are rich in protein that itself has elevated amounts of fixed nitrogen.

Selection – The process by which functional variants in a population of organisms (generated largely through mutation of DNA) thrive (positive selection) or fail (negative selection). Selection shapes the evolutionary history of a population organism since each individual passes on their genome (made of DNA) to their progeny. If an individual thrives because it is better adapted to live in an environment, it will produce more progeny that then outnumber those individuals that are less well adapted to live in an environment. Through multiple iterations of this process, the population is said to have evolved.

Symbiosis – A relationship between two organisms that is beneficial to both partners. An example of this are legumes and their associated *Rhizobia*. The legume (plant) generates specialized root structures called nodules that house *Rhizobia* (Bacteria). The plant provides the *Rhizobia* with organic carbon produced by photosynthesis while the *Rhizobia* provides the plant with fixed N produced by the enzyme nitrogenase.

Trophic Structure – This term refers to the partitioning of biomass among different components of an ecosystem. In photosynthetically supported ecosystems, plants, Cyanobacteria, and algae form the base that secondary and tertiary consumers depend upon for nutrients and energy.