The Microbial World:

The Nitrogen cycle and Nitrogen fixation

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Role of nitrogen in the biosphere

The growth of all organisms depends on the availability of mineral nutrients, and none is more important than nitrogen, which is required in large amounts as an essential component of proteins, nucleic acids and other cellular constituents. There is an abundant supply of nitrogen in the earth's atmosphere - nearly 79% in the form of N_2 gas. However, N_2 is unavailable for use by most organisms because there is a triple bond between the two nitrogen atoms, making the molecule almost inert. In order for nitrogen to be used for growth it must be "fixed" (combined) in the form of ammonium (NH_4) or nitrate (NO_3) ions. The weathering of rocks releases these ions so slowly that it has a neglible effect on the availability of fixed nitrogen. So, nitrogen is often the limiting factor for growth and biomass production in all environments where there is suitable climate and availability of water to support life.

Microorganisms have a central role in almost all aspects of nitrogen availability and thus for life support on earth:

- some bacteria can convert N_2 into ammonia by the process termed **nitrogen fixation**; these bacteria are either free-living or form symbiotic associations with plants or other organisms (e.g. termites, protozoa)
- other bacteria bring about transformations of ammonia to nitrate, and of nitrate to N_2 or other nitrogen gases
- many bacteria and fungi degrade organic matter, releasing fixed nitrogen for reuse by other organisms.

All these processes contribute to the **nitrogen cycle**.

We shall deal first with the process of nitrogen fixation and the nitrogen-fixing organisms, then consider the microbial processes involved in the cycling of nitrogen in the biosphere.

Nitrogen fixation

A relatively small amount of ammonia is produced by lightning. Some ammonia also is produced industrially by the Haber-Bosch process, using an iron-based catalyst, very high pressures and fairly high temperature. But the major conversion of N_2 into ammonia, and thence into proteins, is achieved by microorganisms in the process called nitrogen fixation (or dinitrogen fixation).

The table below shows some estimates of the amount of nitrogen fixed on a global scale. The total biological nitrogen fixation is estimated to be twice as much as the total nitrogen fixation by non-biological processes.

Type of fixation	**N_2 fixed (10^{12} g per year, or 10^6 metric tons per year)**
Non-biological	
Industrial	about 50
Combustion	about 20
Lightning	about 10
Total	**about 80**
Biological	
Agricultural land	about 90
Forest and non-agricultural land	about 50
Sea	about 35
Total	**about 175**
Data from various sources, compiled by DF Bezdicek & AC Kennedy, in *Microorganisms in Action* (eds. JM Lynch & JE Hobbie). Blackwell Scientific Publications 1998.	

To illustrate the importance of biological nitrogen fixation, the image below shows part of the Lower Sonoran desert in Arizona. Every plant that we see in this scene depends ultimately on biological nitrogen fixation. Both free-living cyanobacteria and the cyanobacterial associates of lichens initially contributed nitrogen to the soil by forming a cryptobiotic crust. Now numerous leguminous plants occur in this desert, with nitrogen-fixing *Rhizobium* in their root nodules. Examples are the green-stemmed brush-like trees at the right and left of the image (*Parkinsonia* species, common name "paloverde"), and several acacias and mesquites (not seen in this image).

Mechanism of biological nitrogen fixation

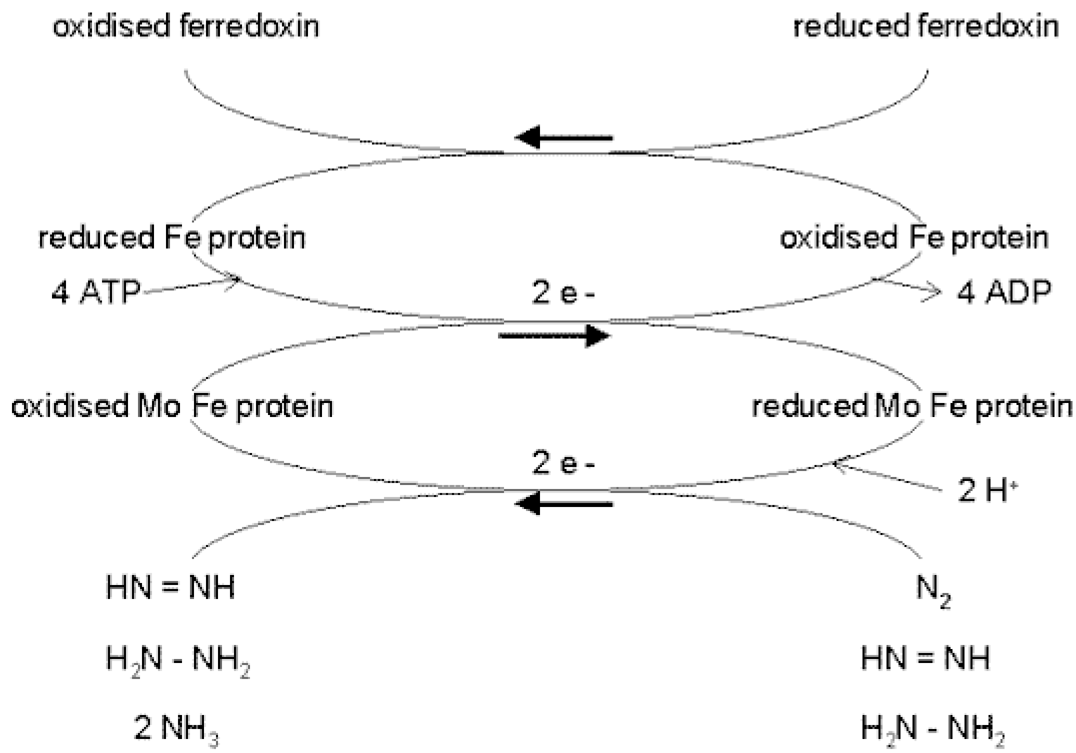
Biological nitrogen fixation can be represented by the following equation, in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen ions):

N_2 + 8H+ + 8e^- + 16 ATP = 2NH_3 + H_2 + 16ADP + 16 Pi

This reaction is performed exclusively by prokaryotes (the bacteria and related organisms), using an enzyme complex termed **nitrogenase**. This enzyme consists of two proteins - an iron protein and a molybdenum-iron protein, as shown below.

The reactions occur while N_2 is bound to the nitrogenase enzyme complex. The Fe protein is first reduced by electrons donated by ferredoxin. Then the reduced Fe protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to H_2N-NH_2, and this in turn is reduced to 2NH_3.

Depending on the type of microorganism, the reduced ferredoxin which supplies electrons for this process is generated by photosynthesis, respiration or fermentation.



There is a remarkable degree of functional conservation between the nitrogenase proteins of all nitrogen-fixing bacteria. The Fe protein and the Mo-Fe protein have been isolated from many of these bacteria, and nitrogen fixation can be shown to occur in cell-free systems in a laboratory when the Fe protein of one species is mixed with the Mo-Fe protein of another bacterium, even if the species are very distantly related.

The nitrogen-fixing organisms

All the nitrogen-fixing organisms are prokaryotes (bacteria). Some of them live independently of other organisms - the so-called free-living nitrogen-fixing bacteria. Others live in intimate symbiotic associations with plants or with other organisms (e.g. protozoa). Examples are shown in the table below.

Examples of nitrogen-fixing bacteria (* denotes a photosynthetic bacterium)			
Free living		Symbiotic with plants	
Aerobic	**Anaerobic** (see Winogradsky column for details)	**Legumes**	**Other plants**
Azotobacter *Beijerinckia* *Klebsiella* (some) Cyanobacteria (some)*	*Clostridium* (some) *Desulfovibrio* Purple sulphur bacteria* Purple non-sulphur bacteria* Green sulphur bacteria*	*Rhizobium*	*Frankia* *Azospirillum*

A point of special interest is that the nitrogenase enzyme complex is highly sensitive to oxygen. It is inactivated when exposed to oxygen, because this reacts with the iron component of the proteins. Although this is not a problem for anaerobic bacteria, it could be a major problem for the aerobic species such as cyanobacteria (which generate oxygen during photosynthesis) and the free-living aerobic bacteria of soils, such as *Azotobacter* and *Beijerinckia*. These organisms have various methods to overcome the problem. For example, *Azotobacter* species have the highest known rate of respiratory metabolism of any organism, so they might protect the enzyme by maintaining a very low level of oxygen in their cells. *Azotobacter* species also produce copious amounts of extracellular polysaccharide (as do *Rhizobium* species in culture - see Exopolysaccharides). By maintaining water within the polysaccharide slime layer, these bacteria can limit the diffusion rate of oxygen to the cells. In the symbiotic nitrogen-fixing organisms such as *Rhizobium*, the root nodules can contain oxygen-scavenging molecules such as **leghaemoglobin**, which shows as a pink colour when the active nitrogen-fixing nodules of legume roots are cut open. Leghaemoglobin may regulate the supply of oxygen to the nodule tissues in the same way as haemoglobin regulates the supply of oxygen to mammalian tissues. Some of the cyanobacteria have yet another mechanism for protecting nitrogenase: nitrogen fixation occurs in special cells (heterocysts) which possess only photosystem I (used to generate ATP by light-mediated reactions) whereas the other cells have both photosystem I and photosystem II (which generates oxygen when light energy is used to split water to supply H_2 for synthesis of organic compounds). Extensive references to these points can be found at: http://chemweb.urich.edu/~sjanson/index.htm (not on this server)

Symbiotic nitrogen fixation

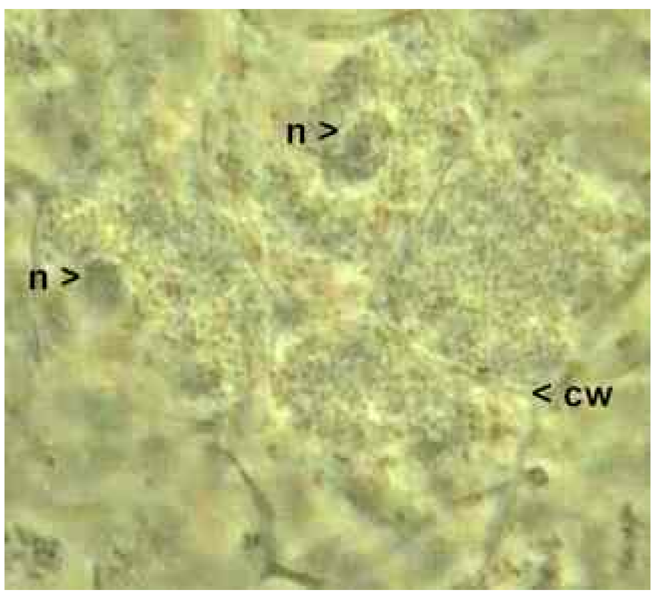
1. Legume symbioses

The most familiar examples of nitrogen-fixing symbioses are the **root nodules of legumes** (peas, beans, clover, etc.).

Part of a clover root system bearing naturally occurring nodules of *Rhizobium*. Each nodule is about 2-3 mm long.

Clover root nodules at higher magnification, showing two partly crushed nodules (arrowheads) with pink-coloured contents. This colour is caused by the presence of the pigment **leghaemoglobin** - a unique metabolite of this type of symbiosis. Leghaemoglobin is found only in the nodules and is not produced by either the bacterium or the plant when grown alone.

In these leguminous associations the bacteria usually are *Rhizobium* species, but the root nodules of soybeans, chickpea and some other legumes are formed by small-celled rhizobia termed *Bradyrhizobium*. Nodules on some tropical leguminous plants are formed by yet other genera. In all cases the bacteria "invade" the plant and cause the formation of a nodule by inducing localised proliferation of the plant host cells. Yet the bacteria always remain separated from the host cytoplasm by being enclosed in a membrane - a necessary feature in symbioses (see the image below).



Part of a crushed root nodule of a pea plant, showing four root cells containing colonies of *Rhizobium*. The nuclei (**n**) of two root cells are shown; **cw** indicates the cell wall that separates two plant cells. Although it cannot be seen clearly in this image, the bacteria occur in clusters which are enclosed in membranes, separating them from the cytoplasm of the plant cells.

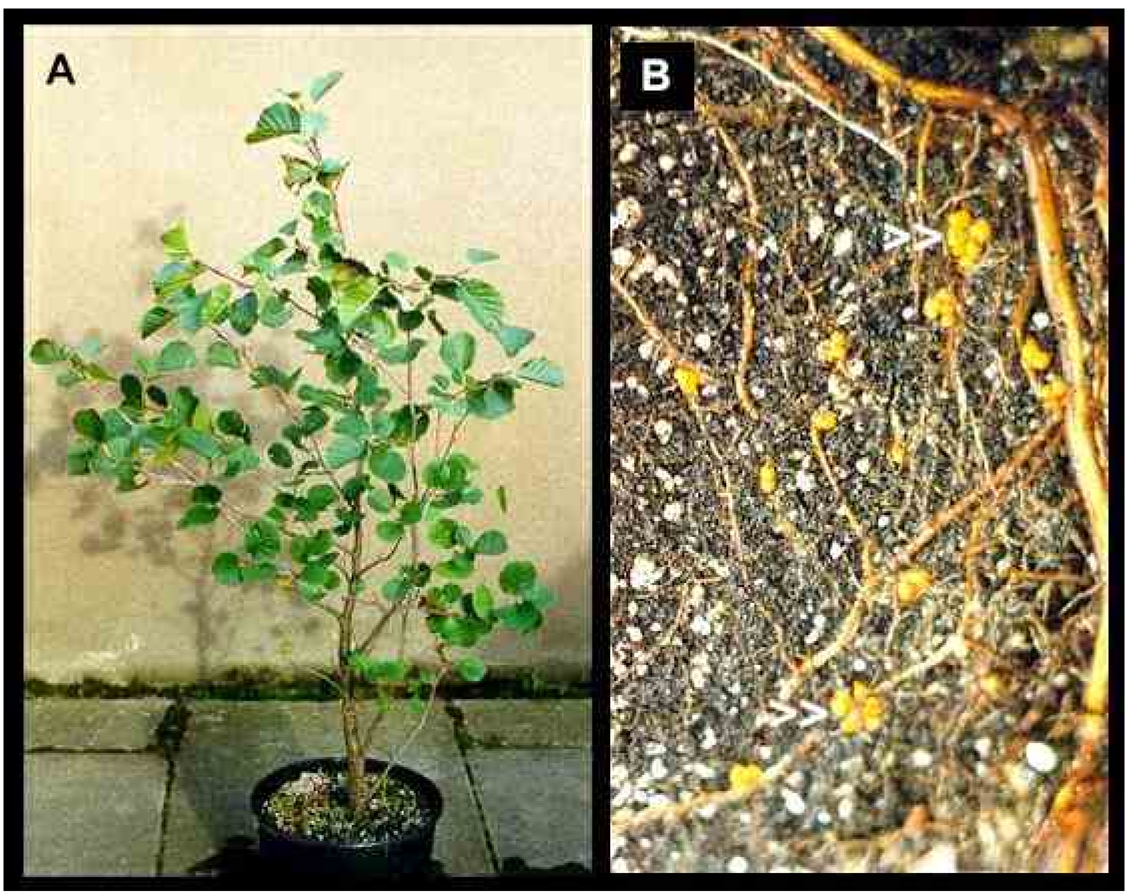
In nodules where nitrogen-fixation is occurring, the plant tissues contain the oxygen-scavenging molecule, **leghaemoglobin** (serving the same function as the oxygen-carrying haemoglobin in blood). The function of this molecule in nodules is to reduce the amount of free oxygen, and thereby to protect the nitrogen-fixing enzyme **nitrogenase**, which is irreversibly inactivated by oxygen.

Some excellent images and discussion of these leguminous associations can be found at:

CIFN/UNAM Brochure - Research (not on this server)

2. Associations with *Frankia*

Frankia is a genus of the bacterial group termed **actinomycetes** - filamentous bacteria that are noted for their production of air-borne spores. Included in this group are the common soil-dwelling *Streptomyces* species which produce many of the antibiotics used in medicine (see *Streptomyces*). *Frankia* species are slow-growing in culture, and require specialised media, suggesting that they are specialised symbionts. They form nitrogen-fixing root nodules (sometimes called actinorhizae) with several woody plants of different families, such as alder (*Alnus* species), sea buckthorn (*Hippophae rhamnoides*, which is common in sand-dune environments) and *Casuarina* (a Mediterranean tree genus). Figure A (below) shows a young alder tree (*Alnus glutinosa*) growing in a plant pot, and Figure B shows part of the root system of this tree, bearing the orange-yellow coloured nodules (arrowheads) containing *Frankia.*



Alder and the other woody hosts of *Frankia* are typical pioneer species that invade nutrient-poor soils. These plants probably benefit from the nitrogen-fixing association, while supplying the bacterial symbiont with photosynthetic products.

3. Cyanobacterial associations

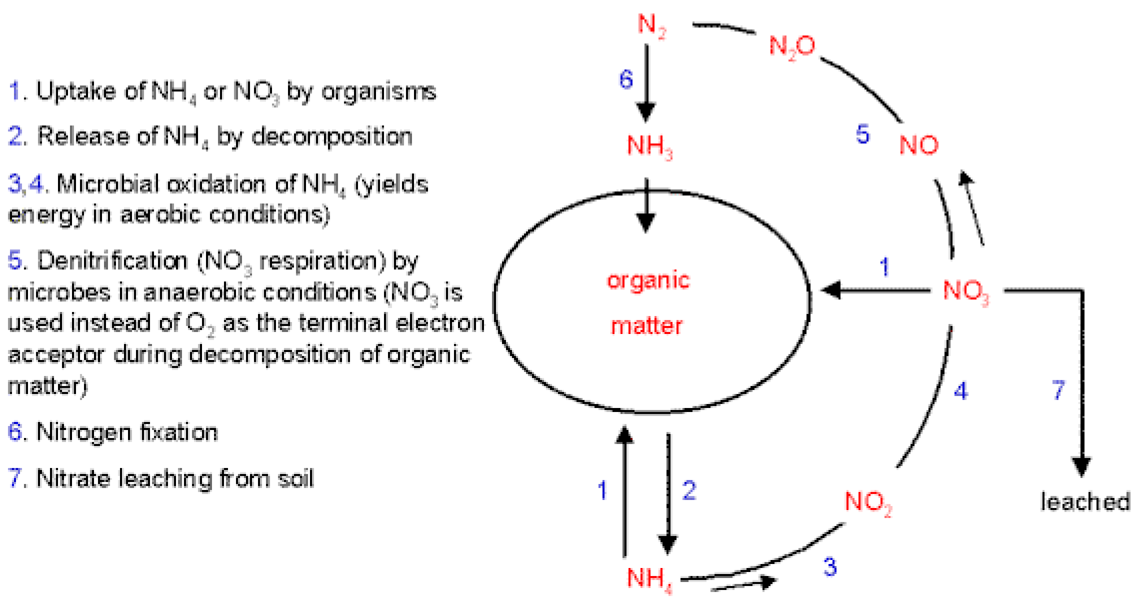
The photosynthetic cyanobacteria often live as free-living organisms in pioneer habitats such as desert soils (see cyanobacteria) or as symbionts with lichens in other pioneer habitats. They also form symbiotic associations with other organisms such as the water fern *Azolla*, and cycads.The association with *Azolla*, where cyanobacteria (*Anabaena azollae*) are harboured in the leaves, has sometimes been shown to be important for nitrogen inputs in rice paddies, especially if the fern is allowed to grow and then ploughed into the soil to release nitrogen before the rice crop is sown. A symbiotic association of cyanobacteria with cycads is shown below. The first image shows a pot-grown plant. The second image shows a close-up of the soil surface in this pot. Short, club-shaped, branching roots have grown into the aerial environment. These aerial roots contain a nitrogen-fixing cyanobacterial symbiont.

In addition to these intimate and specialised symbiotic associations, there are several free-living nitrogen-fixing bacteria that grow in close association with plants. For example, *Azospirillum* species have been shown to fix nitrogen when growing in the root zone (rhizosphere) or tropical grasses, and even of maize plants in field conditions. Similarly, *Azotobacter* species can fix nitrogen in the rhizosphere of several plants. In both cases the bacteria grow at the expense of sugars and other nutrients that leak from the roots. However, these bacteria can make only a small contribution to the nitrogen nutrition of the plant, because nitrogen-fixation is an energy-expensive process, and large amounts of organic nutrients are not continuously available to microbes in the rhizosphere.

This limitation may not apply to the bacteria that live in root nodules or other intimate symbiotic associations with plants. It has been estimated that nitrogen fixation in the nodules of clover roots or other leguminous plants may consume as much as 20% of the total photosynthate.

The nitrogen cycle

The diagram below shows an overview of the nitrogen cycle in soil or aquatic environments. At any one time a large proportion of the total fixed nitrogen will be locked up in the biomass or in the dead remains of organisms (shown collectively as "organic matter"). So, the only nitrogen available to support new growth will be that which is supplied by nitrogen fixation from the atmosphere (pathway 6 in the diagram) or by the release of ammonium or simple organic nitrogen compounds through the decomposition of organic matter (pathway 2). Some of other stages in this cycle are mediated by specialised groups of microorganisms and are explained below.



Nitrification

The term nitrification refers to the conversion of ammonium to nitrate (pathway 3-4). This is brought about by the **nitrifying bacteria**, which are specialised to gain their energy by oxidising ammonium, while using CO_2 as their source of carbon to synthesise organic compounds. Organisms of this sort are termed **chemoautotrophs** - they gain their energy by chemical oxidations (chemo-) and they are autotrophs (self-feeders) because they do not depend on pre-formed organic matter. In principle the oxidation of ammonium by these bacteria is no different from the way in which humans gain energy by oxidising sugars. Their use of CO_2 to produce organic matter is no different in principle from the behaviour of plants.

The nitrifying bacteria are found in most soils and waters of moderate pH, but are not active in highly acidic soils. They almost always are found as mixed-species communities (termed **consortia**) because some of them - e.g. *Nitrosomonas* species - are specialised to convert ammonium to nitrite (NO_2^-) while others - e.g. *Nitrobacter* species - convert nitrite to nitrate (NO_3^-). In fact, the accumulation of nitrite inhibits *Nitrosomonas*, so it depends on *Nitrobacter* to convert this to nitrate, whereas *Nitrobacter* depends on *Nitrosomonas* to generate nitrite.

The nitrifying bacteria have some important environmental consequences, because they are so common that most of the ammonium in oxygenated soil or natural waters is readily converted to nitrate. Most plants and microorganisms can take up either nitrate or ammonium (arrows marked "1" in the diagram). However, process of nitrification has some undesirable consequences. The ammonium ion (NH_4^+) has a positive charge and so is readily adsorbed onto the negatively charged clay colloids and soil organic matter, preventing it from being washed out of the soil by rainfall. In contrast, the negatively charged nitrate ion is not held on soil particles and so can be washed down the soil profile - the process termed leaching (arrow marked 7 in the diagram). In this way, valuable nitrogen can be lost from the soil, reducing the soil fertility. The nitrates can then accumulate in groundwater, and ultimately in drinking water. There are strict regulations governing the amount of nitrate that can be present in drinking water, because nitrates can be reduced to highly reactive nitrites by microorganisms in the anaerobic conditions of the gut. Nitrites are absorbed from the gut and bind to haemoglobin, reducing its oxygen-carrying capacity. In young babies this can lead to respiratory distress - the condition known as "blue baby syndrome". Nitrite in the gut also can react with amino compounds, forming highly carcinogenic nitrosamines.

Denitrification

Denitrification refers to the process in which nitrate is converted to gaseous compounds (nitric oxide, nitrous oxide and N_2) by microorganisms. The sequence usually involves the production of nitrite (NO_2^-) as an intermediate step is shown as "5" in the diagram above. Several types of bacteria perform this conversion when growing on organic matter in anaerobic conditions. Because of the lack of oxygen for normal aerobic respiration, they use nitrate in place of oxygen as the terminal electron acceptor. This is termed anaerobic respiration and can be illustrated as follows:

In aerobic respiration (as in humans), organic molecules are oxidised to obtain energy, while oxygen is reduced to water:

$C_6H_{12}O_6$ + 6 O_2 = 6 CO_2 + 6 H_2O + energy

In the absence of oxygen, any reducible substance such as nitrate (NO_3^-) could serve the same role and be reduced to nitrite, nitric oxide, nitrous oxide or N_2.

Thus, the conditions in which we find denitrifying organisms are characterised by (1) a supply of oxidisable organic matter, and (2) absence of oxygen but availability of reducible nitrogen sources. A mixture of gaseous nitrogen products is often produced because of the stepwise use of nitrate, nitrite, nitric oxide and nitrous oxide as electron acceptors in anaerobic respiration. The common denitrifying bacteria include several species of *Pseudomonas*, *Alkaligenes* and *Bacillus*. Their activities result in substantial losses of nitrogen into the atmosphere, roughly balancing the amount of nitrogen fixation that occurs each year.

Further information: websites

International Symbiosis Society (not on this server)

A valuable site that deals with the ecology and physiology of N fixation: http://web.reed.edu/academic/departments/biology/nitrogen/ (not on this server)

A research-related site on Molecular genetics of plant-microbe interactions: http://www.lifesci.ucla.edu/mcdbio/html/ri4.htm (not on this server)

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