

## **CHAPTER-7**

### **BIOCHEMICAL TRANSFORMATIONS OF NITROGEN**

Perhaps more time and effort have been invested in studying the nitrogen cycle than any other topic in soil microbiology. Nitrogen is an essential nutrient for all life on earth. Thus its fixation into usable forms by bacteria and subsequent transformations and recycling through organic and inorganic forms are of great practical interest. Indeed, nitrogen is the nutrient most often limiting plant growth in terrestrial ecosystems. The nitrogen cycle affects the environment as well. Current concerns include high concentrations of nitrate in ground and surface waters and the contribution of gaseous nitrogen oxides, such as NO and N<sub>2</sub>O to large-scale environmental problems of acid rain, ozone depletion, and greenhouse warming. The large diversity of nitrogen containing compounds, which exist in numerous oxidation states, and the wide array of microbial transformations makes the nitrogen cycle an extremely interesting intellectual challenge.

#### **The Nitrogen Cycle**

An overview of the nitrogen cycle is presented in figure 1. Nitrogen is present in various forms primarily as dinitrogen gas (N<sub>2</sub>), organic nitrogen (in plants, animals, microbial biomass, and soil organic matter), and, ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) ions. Microbially mediated processes transform nitrogen from one form to another. Certain bacteria can transform dinitrogen to ammonia (NH<sub>3</sub>) by a process known as N<sub>2</sub> fixation. The process of ammonification/immobilization, nitrification, and denitrification are responsible for moving the fixed nitrogen from one form to another in the soil and will be discussed in turn in this chapter.

#### **Forms of Nitrogen:**

The sizes of the nitrogen pools vary over several orders of magnitude. Although we tend to ignore the relatively inert dinitrogen pool, probably because it is an invisible gas, it actually represents the largest pool of biologically active nitrogen in terrestrial ecosystem. Soil organic nitrogen makes up the next largest pool of nitrogen and varies widely among soil types. The variation in soil organic nitrogen is determined largely by the factors of soil formation, particularly temperature and precipitation.

The amount of nitrogen tied up in plant biomass is of intermediate size and varies as a function of vegetation type (forests versus grasslands), climate, and soil nitrogen availability. Soil inorganic-nitrogen pools are usually small, generally just a few mg N kg<sup>-1</sup> in natural ecosystem and rarely exceeding 100 mg N kg<sup>-1</sup> in the plow layer of recently fertilized agricultural soils.

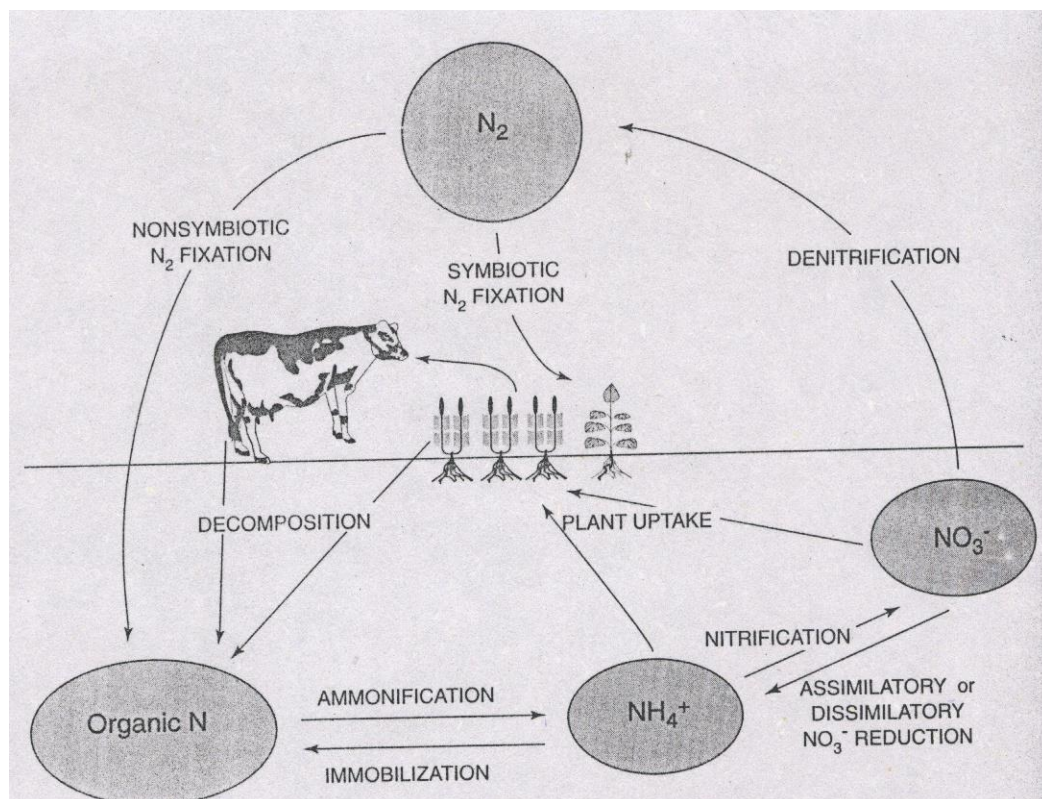


Figure 1: Nitrogen Cycle

Larger pools tend to be the less reactive (i.e., they turn over more slowly) and the smaller pools usually are more dynamic. For example, the atmospheric dinitrogen pool is the largest pool of nitrogen and has a mean residence time on the order of thousands to millions of years. Decades are required to turn over the organic-nitrogen pool. Nitrogen in plant biomass often turns over annually, whereas inorganic-nitrogen pools are so dynamic that they may turn over more than once a day.

**Soil organic nitrogen:** The nitrogen contained in soil organic matter occurs in a wide range of compounds, of which only about half can be definitively identified. Naturally occurring organic-nitrogen compounds isolated from soils include: proteins and amino acids, microbial cell-wall polymers and amino sugars, nucleic acids, and a verity of vitamins, antibiotics, and metabolic intermediates. Because much of the organic nitrogen in soil is of unknown composition, a fractionation procedure based on acid hydrolysis has been used to characterize soil organic nitrogen. It is interesting to note that the range given for amino-sugar nitrogen, which is found mainly in microbial cell walls, is similar to that often found for microbial biomass nitrogen, which is typically about 5% of total soil nitrogen.

**Soil inorganic nitrogen:** Unlike soil organic nitrogen, the important inorganic forms of nitrogen in soil ecosystems are well characterized, primarily because most inorganic-nitrogen compounds can be readily separated and measured. Inorganic-nitrogen pools in soil are usually small compared to organic nitrogen, but are nevertheless important because they serve as substrates, metabolic intermediates, alternate electron acceptors, or products of the many biological nitrogen transformations. Some key inorganic nitrogen compounds include Ammonium ( $\text{NH}_4^+$ , oxidation number -3), Hydroxylamine ( $\text{NH}_2\text{OH}$ , oxidation number -1), Dinitrogen ( $\text{N}_2$ , oxidation number -0), Nitrous oxide ( $\text{N}_2\text{O}$ , oxidation number +1), Nitric oxide ( $\text{NO}$ , oxidation number +2), Nitrite ( $\text{NO}_2^-$ , oxidation number +3), Nitrate ( $\text{NO}_3^-$ , oxidation number +5)

### **NITROGEN MINERALIZATION (AMMONIFICATION)/IMMOBILIZATION**

Nitrogen mineralization has several meanings. It is sometimes used in a generic sense for the production of inorganic nitrogen, both ammonium and nitrate, and sometimes more narrowly for the production of ammonium. The increase (or sometimes decrease) in inorganic nitrogen is most often called *net nitrogen mineralization* because it represents the sum of the concurrent ammonium production and consumption processes. It is more correct to use **ammonification**, or *gross nitrogen mineralization*, to describe the biological transformation of organic nitrogen to ammonium.

Less confusion surrounds the term **immobilization** because it almost always describes the conversion of ammonium to organic nitrogen, primarily as a result of the assimilation of ammonium by the microbial biomass, a process which temporarily renders the nitrogen unavailable for plants or microbes. Less frequently, immobilization may refer to the assimilation of both ammonium and nitrate. The assimilation of nitrate by the microbial biomass is usually specified explicitly as nitrate immobilization. It is important to remember, however, that nitrate assimilation requires that nitrate be reduced to ammonium before the nitrogen can be incorporated into cell constituents.

#### **Ammonification**

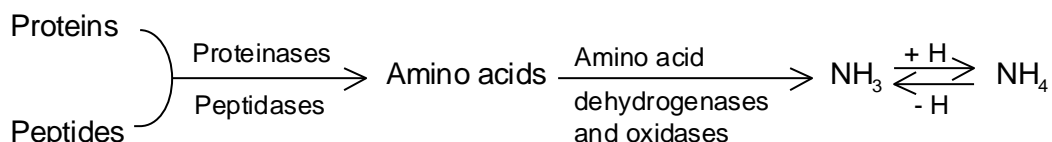
The conversion of organic-nitrogen compounds to ammonium is mediated by enzymes produced by microbes and soil animals. Production of ammonium often involves several steps. Extracellular enzymes first break down organic-nitrogen polymers and the resulting monomers pass across the cell membrane and are further metabolized, with the resulting production of ammonium, which is released into the soil solution. The major extracellular enzymes produced

by microorganisms depolymerize proteins, amino-polysaccharides (microbial cell walls), and nucleic acids and hydrolyze urea.

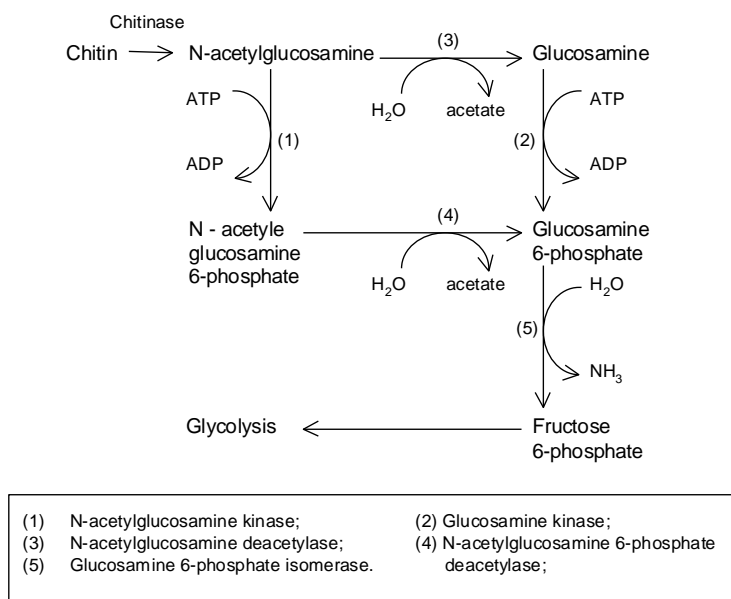
**Table: Extracellular enzymes involved in microbial nitrogen mineralization**

Substrate	Enzyme	Product
Protein	Proteinases, proteases	Peptides, amino acids
Peptides	Peptidases	Amino acids
Chitin	Chitinases	Chitobiose
Chitobiose	Chitobiase	N-acetylglucosamine
Peptidoglycan	Lysozyme	N-acetylglucosamine, N-acetylmuramic acid
DNA & RNA	Endonucleases and exonucleases	Nucleotides
Urea	Urease	NH <sub>3</sub> , CO <sub>2</sub>

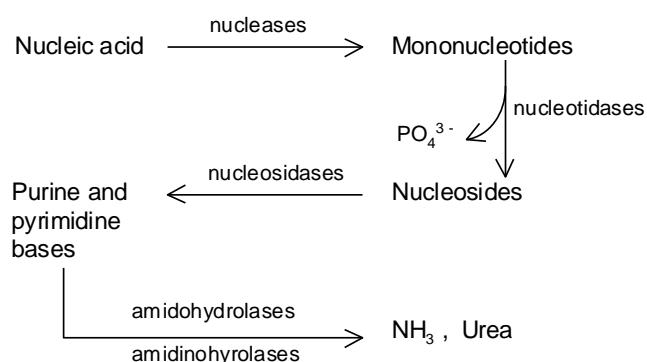
Proteins are broken down by a wide variety of proteinases, also called proteases, and peptidases. Proteinases work on large proteins whereas peptidases may cleave dipeptides or split off an individual amino acid. The enzymes are classified according to their active site and substrate specificity, but all hydrolytically cleave peptide bonds to ultimately produce individual amino acids. Examples of proteolytic enzymes isolated from soil microbes include subtilisin, clostripain, and thermolysin.



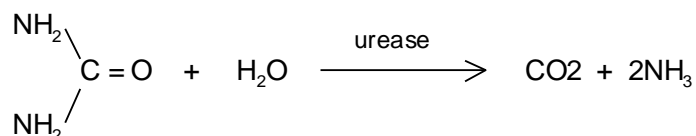
Although microbial cell walls are thought to be relatively recalcitrant in soils, several common extracellular enzymes will degrade these polymers. Chitin, which forms the cell wall of many fungi and is also part of insect exoskeletons, is degraded by the combined activities of chitinase and chitobiase. Chitinase breaks chitin, a polymer of N-acetylglucosamine, into dimers (chitobiose), which are subsequently cleaved to two molecules of N-acetylglucosamine by chitobiase. This process is analogous to the enzymatic degradation of cellulose. Several enzymes work to degrade the peptidoglycan portion of bacterial cell walls. Lysozyme is perhaps the most well known. It breaks the  $\beta$  1, 4 linkage between N-acetylmuramic acid and N-acetylglucosamine. Individual amino-sugar monomers are the end products of the extracellular enzymes that degrade microbial cell walls.



Nucleic acids are degraded by ribonucleases (RNases) and deoxyribonucleases (DNases), which hydrolyze the ester bonds between the phosphate groups and pentose sugars of nucleic acids. The known types of RNases and DNases are divided into exonucleases, which split off a single nucleotide from the end of the nucleic acid polymer, or endonucleases, which cleave within the nucleic acid polymer. Individual nucleotides are the ultimate product of the nucleases. The nucleotides may give rise to NH<sub>3</sub> and urea:



Urease is another important extracellular enzyme involved in ammonification. Ureases hydrolyze urea into carbon dioxide and ammonia. Nickel is the cofactor associated with the active site of at least some ureases. Ureases function in the utilization of natural sources of urea (e.g., animal wastes) but perhaps most importantly in making the nitrogen in urea fertilizer available to plants.



In most cases, the final production of ammonium occurs within microbial cells through the action of intracellular enzymes. Of course, some of these intracellular enzymes may become “extracellular” when microbial cells are lysed.

Two types of nitrogen are found in amino acids: the amine ( $\text{NH}_2\text{-CR}_3$ ) and amide ( $\text{NH}_2\text{-CR=O}$ ) functional groups. The amide groups of asparagine and glutamine are cleaved by asparaginase and glutaminase. Amino nitrogen is released primarily by amino-acid dehydrogenases and amino-acid oxidases in a process known as *deamination*. Dehydrogenases, such as glutamate dehydrogenase, use NAD as a factor to accept electrons.

Amino sugars are metabolized in two steps. First, the amino sugar is phosphorylated by a *kinase* and then ammonia is released through a deamination reaction.

Degradation of nucleotides and the release of ammonium typically require several steps. First nucleotides are hydrolyzed to produce nucleosides and  $\text{PO}_4^{3-}$ . Following the dephosphorylation, the nucleosides are further hydrolyzed to purine or pyrimidine bases and pentose sugar moieties. Normal metabolic pathways then release ammonium during the catabolism of purines and pyrimidines, with urea as a prominent intermediate.

In most instances the microbial degradation of amino acids, amino sugars, and nucleic acids is driven by the need of heterotrophic microbes for energy and carbon. Thus, the ammonium released as a result of ammonification can be considered a by product of catabolism. At least in pure culture studies, microbes grow better with a carbohydrate as a carbon and energy source and ammonium or nitrate as a source of nitrogen than if grown on organic-nitrogen compounds alone.

The common mechanisms for initial degradation of amino acids are deamination, the removal of ammonia, and decarboxylation, in which carboxyl is removed.

- a) Deamination by direct removal of ammonia:  
 $\text{RCH}_2\text{CHNH}_2\text{COOH} \longrightarrow \text{RCH=CHCOOH} + \text{NH}_3$
- b) Oxidative deamination:  
 $\text{RCHNH}_2\text{COOH} + \frac{1}{2}\text{O}_2 \longrightarrow \text{RCOCOOH} + \text{NH}_3$
- c) Reductive deamination:  
 $\text{RCHNH}_2\text{COOH} + 2\text{H} \longrightarrow \text{RCH}_2\text{COOH} + \text{NH}_3$
- c) Decarboxylation:  
 $\text{RCHNH}_2\text{COOH} \longrightarrow \text{RCH}_2\text{NH}_2 + \text{CO}_2$

## IMMOBILIZATION

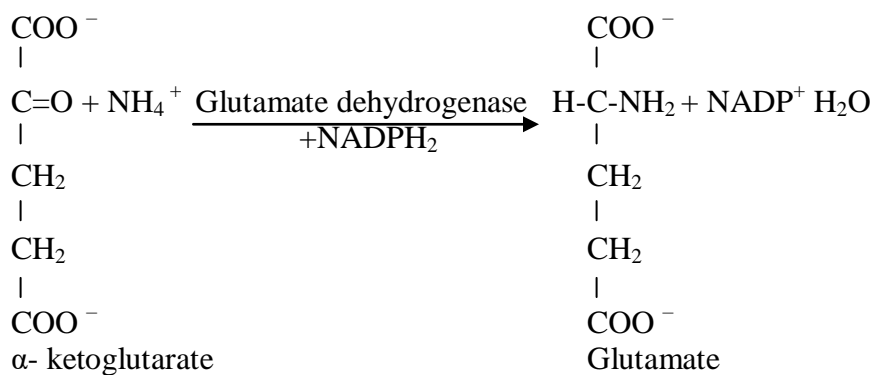
Immobilization (or assimilation-the two are synonymous terms) is the incorporation of inorganic N into organic N by soil microorganisms. The N is immobilized with respect to plants; if it is being turned into new microbial cells, it cannot be taken up by plants until those microbial cells

decompose or mineralize. The consequences of inadequate N in soil can be devastating for plants.

### **NH<sub>4</sub><sup>+</sup> Assimilation**

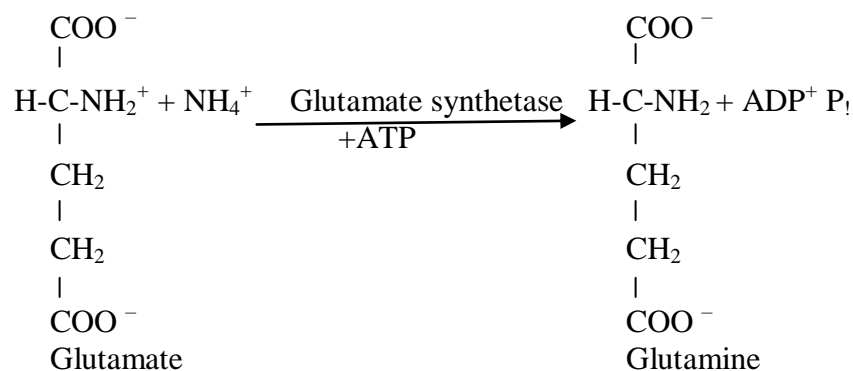
There are two major pathways in which NH<sub>4</sub><sup>+</sup> ions are assimilated by microorganisms. The first NH<sub>4</sub><sup>+</sup> assimilation pathway is directly into glutamate by the enzyme glutamate dehydrogenase (GDH). This pathway operates at relatively high NH<sub>4</sub><sup>+</sup> concentrations (> 1 mM in solution or >0.5 mg NH<sub>4</sub><sup>+</sup>/kg soil).

At lower NH<sub>4</sub><sup>+</sup> concentration (<1 mM NH<sub>4</sub><sup>+</sup> or <0.1 mg NH<sub>4</sub><sup>+</sup>/kg soil) another pathway is used- GS/GOGAT. (GS= glutamine synthetase; GOGAT = glutamine α-oxoglutarate amino transferase [Glutamate Synthase]). It is a two-step process in which the primary assimilation of NH<sub>4</sub><sup>+</sup> occurs in a reaction catalyzed by GS. The second step in the process forms glutamate from the transamination of α- ketoglutarate by glutamine. Glutamine synthetase –catalysed reactions require ATP, but the affinity of GS for NH<sub>4</sub><sup>+</sup> is greater than that of GDH. This means that GS allows microorganisms to utilize lower concentrations of environmental NH<sub>4</sub><sup>+</sup>. When both pathways are present, GS is repressed by high NH<sub>4</sub><sup>+</sup> concentrations. Once N is incorporated as glutamate, it is transferred via transamination reactions to keto acids to form amino acids.



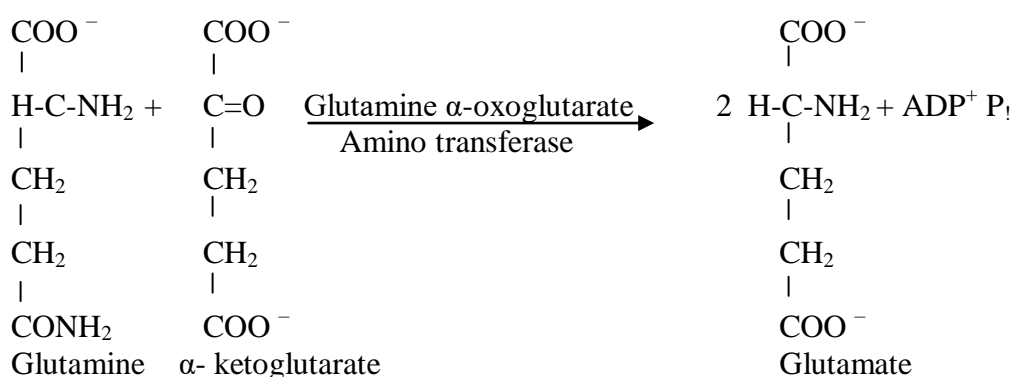

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Reductive amination of α- ketoglutarate by glutamate dehydrogenase-a mechanism of NH<sub>4</sub><sup>+</sup> assimilation



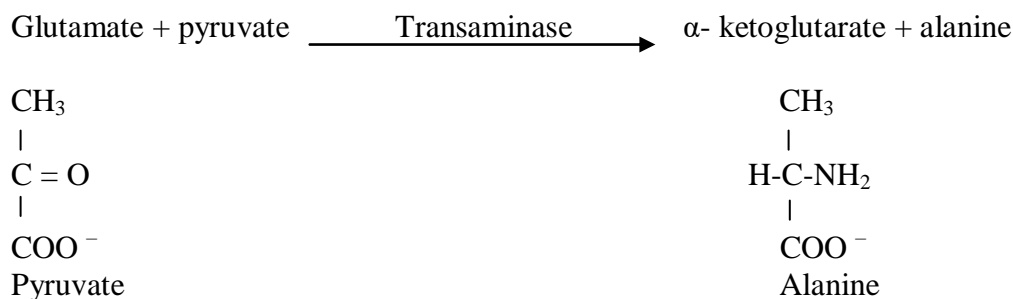

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Glutamine formation-the first step in  $\text{NH}_4^+$  assimilation by GS/GOGAT




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Glutamate formation-the second step in  $\text{NH}_4^+$  assimilation by GS/GOGAT




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Immobilization of assimilated N into amino acids

### **$\text{NO}_3^-$ Assimilation**

Many bacteria and fungi assimilate inorganic N as  $\text{NO}_3^-$ . To do this, they first reduce the  $\text{NO}_3^-$  to  $\text{NO}_2^-$  using an assimilatory  $\text{NO}_3^-$  reductase enzyme. Then they reduce the  $\text{NO}_2^-$  to  $\text{NH}_4^+$  via an enzyme called assimilatory  $\text{NO}_2^-$  reductase. Assimilatory  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reductases are synthesized only when soil N is limiting or when  $\text{NO}_3^-$  is present. Compounds such as  $\text{NH}_4^+$ , glutamine, and glutamate are all preferentially used by soil microorganisms. Even when microbial cells have synthesized assimilatory  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reductase and are actively taking up



$\text{NO}_3^-$ , that uptake can be almost immediately inhibited by adding  $\text{NH}_4^+$  to the environment. This inhibition could simply be because the  $\text{NH}_4^+$  inhibited  $\text{NO}_3^-$  uptake, or because it suppressed either the synthesis or activity of  $\text{NO}_3^-$  assimilating enzymes.

Adding  $\text{NH}_4^+$  has an almost immediate inhibitory effect (<1 minute) on  $\text{NO}_3^-$  uptake. The mechanism of inhibition is by preventing  $\text{NO}_3^-$  from getting into the microbial cells, because an effect on enzyme synthesis wouldn't have been nearly as rapid. Compounds such as glutamine and asparagine have almost as great an inhibitory effect on  $\text{NO}_3^-$  uptake as  $\text{NH}_4^+$ , but takes longer to cause the inhibition (> 4 minutes). Once the  $\text{NO}_3^-$  is reduced to  $\text{NH}_4^+$ , it is probably via the GS/GOGAT pathway of N assimilation rather than by GDH (glutamate dehydrogenase).

### **N Availability**

Mineralization and immobilization occur simultaneously. Organic N and  $\text{NH}_4^+$  are continuously cycling back and forth. Whether mineralization or immobilization occurs during decomposition of plant residue depends on the C: N content of the material being decomposed by heterotrophic organisms.

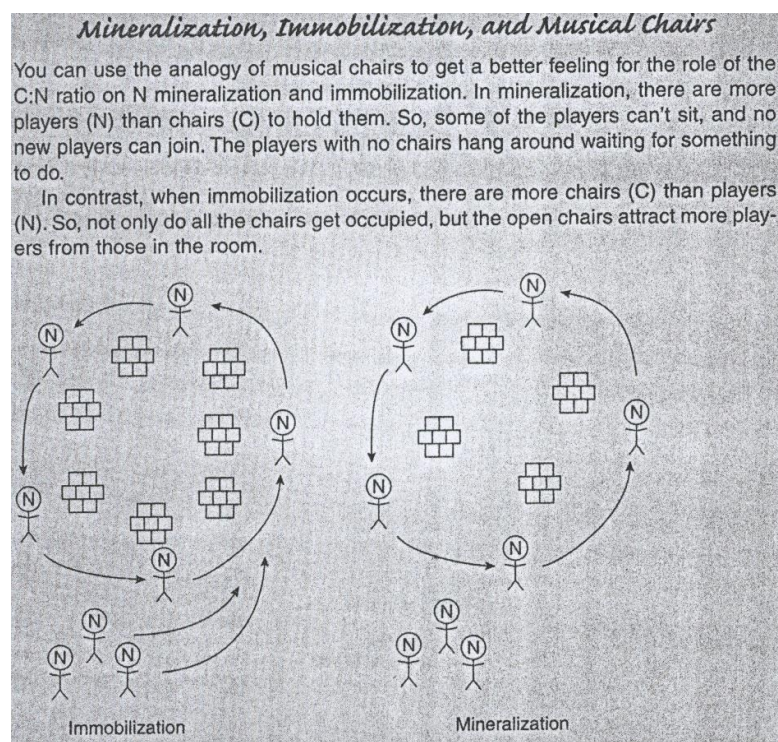
The C: N ratio reflects the relative proportion of C and N in a given substance. More importantly, in microorganisms it indicates approximately how much N must be assimilated for every gram of C that is converted into biomass. Soil bacteria usually have a C: N ratio of 5:1 to 8:1. This is because microbial cell walls are about 45% C and 6 to 9% N. However, the C: N ratio of microorganisms is not constant. Fungi have a wide C: N ratio i.e. 4.5:1 to 15:1 because some fungi, especially phycomycetes, contain cellulose in their cell wall. Many bacteria produce polysaccharides under N-limitation that will raise their C: N ratio.

When a substance entering soil has a high C: N ratio (40:1 for example), net immobilization occurs because there is not enough N availability in the substance to convert the entire C into biomass. To make up the deficit, microorganisms in the immediate environment assimilate the entire available inorganic N in their vicinity, which makes it unavailable for plants.

At low C: N ratios (20:1 for example), net mineralization occurs. There is enough N in the substrate for the microorganisms to convert the C into biomass and the excess N accumulates in soil. The decomposition rate of added material is affected by the availability of N in plant and soil and fertilizer. The "priming effect" is a stimulated decomposition of existing soil organic material by the addition of fresh substrates. The more microorganisms that can grow on a substrate, the greater the microbial biomass that will be formed, the greater will be the extent of decomposition that can occur. As plant decomposition proceeds, C: N ratio falls due to loss of C

through respiration while cellular N is retained. Eventually, the C:N ratios of the decomposing material will approximate the C: N ratio of the resident microbial population.

The C: N ratio is a good, but not absolute, indicator of whether N immobilization is likely to happen. It depends, in part, on the availability of C. Trees can have a C: N ratio  $> 50:1$  and still be mineralized because the effective C: N ratio of their tissue is lower by virtue of the lignin content. The lignin is virtually insoluble and indigestible by most microorganisms, so for all intents and purpose, it has no effect on metabolism of other substrates. As plants age, the lignin content goes up as well as the C: N ratio. Remember, mineralization rates are not constant because the availability of all mineralizable substrate is not constant. As a general rule, 1 kg of N is required to decompose every 100 kg of dry plant material with a low N content, such as straw.



### Fate of Ammonium in Soil

In addition to the mineralization/immobilization cycle, ammonium has several other fates in soil. It can be chemically held on cation exchange sites or become fixed in the lattice of clay minerals (ammonium fixation), such as illite and vermiculite. Ammonium may react chemically with organic compounds, such as quinines, or it may be volatilized at high pH. Major biological fates are plant uptake, microbial assimilation, or oxidation to nitrate by nitrifying microorganisms.

## NITRIFICATION

Nitrification is the microbial production of nitrate from the oxidation of reduced nitrogen compounds. Most often we think of autotrophic nitrification, the two-step, two-organism process of oxidizing ammonium to nitrate, in which the inorganic nitrogen serves as the energy source for the nitrifying bacteria. The first step of autotrophic nitrification is ammonia oxidation, the conversion of ammonium (actually, ammonia at the enzyme level) to nitrate by the ammonia-oxidizing bacteria of the “Nitroso-” genera. Nitrite is then oxidized to nitrate by the nitrate-oxidizing bacteria of the “Nitroso-” genera.

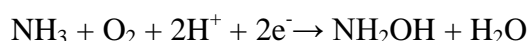
### Ammonia Oxidation

Ammonia-oxidizing bacteria are throughout to be a relatively defined and coherent group. Representatives from each of the five genera of ammonia oxidizers have been isolated from soil. Although *Nitrosomonas* has been the best characterized and most well studied ammonia oxidizer, particularly with respect to its enzymology and the biochemistry of ammonia oxidation, *Nitrosolobus* is thought to be the dominant ammonia oxidizer in many soils. *Nitrosopira* is associated with acid soils.

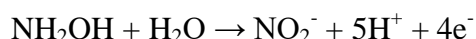
The overall reaction for the conversion of ammonia nitrate is:



This oxidation is a  $6e^-$  transfer that yields 271 kJ (65-kcal)  $\text{mol}^{-1}$   $\text{NH}_3$ . The first step in the reaction is the conversion of  $\text{NH}_3$  to  $\text{NH}_2\text{OH}$  (hydroxylamine) by the membrane-bound *ammonia monooxygenase enzyme*:



This reaction is endergonic and requires a small amount of energy. Hydroxylamine is converted through several undefined steps to nitrite with an overall reaction of:



This is an energy-yielding reaction, with two of the electron produced passing down the electron-transport chain to oxygen while the other two are used in the ammonia monooxygenase reaction. The initial step of this reaction is catalyzed by hydroxylamine oxidoreductase, a soluble enzyme. The nitroxyl radical ( $\text{HNO}$ ) is thought to be produced from the oxidation of  $\text{NH}_2\text{OH}$  and may be the source of some of the nitric oxide ( $\text{NO}$ ) that is released as a by-product of nitrification. The final step(s) in the production of nitrate are not well-defined.

Two other products of ammonia oxidation are nitrous oxide ( $\text{N}_2\text{O}$ ) and acidity. Ammonia oxidizers contain a nitrite reductase, which is capable of reducing  $\text{NO}_2^-$  to  $\text{N}_2\text{O}$ . Under aerobic

conditions, the production of nitrous oxide by this mechanism is small, less than 1% of the ammonia oxidized. As oxygen availability decreases, however, relatively more nitrous oxide is produced as nitrite is used as the electron acceptor. In some habitats, nitrification may be a major source of gaseous nitrogen oxides.

Ammonia oxidation acidifies by releasing one mole of  $\text{H}^+$  for every mole of ammonia oxidized. This presents a paradox, as nitrifying bacteria generally grow best at neutral pH and their activity is often inhibited by low pH. Nevertheless, the production of acidity by ammonia oxidizers has been shown to be responsible for lowering the pH of natural and agricultural soils.

### Nitrite Oxidation

Nitrite-oxidizing bacteria are phylogenetically more diverse than the ammonia oxidizers. Most soil isolates are *Nitrobacter* spp., although a *Nitrospira* strain has also been isolated from soil.

The oxidation of nitrite to nitrate is a one-step reaction,:



Nitrite is oxidized to nitrate by a membrane-bound *nitrite oxidoreductase*, which transfer oxygen from water and transfers a pair of electrons to the electron-transport chain for the production of ATP via oxidative phosphorylation:



This reaction yields 77 kJ (918 kcal)  $\text{mol}^{-1}$  nitrite utilized, about one-third that of ammonia oxidation. Nitrite oxidation can be competitively inhibited by chlorate ( $\text{ClO}_4^-$ ), which is useful in experimental studies to determine rates of autotrophic versus heterotrophic nitrification.

Unlike ammonia oxidizers, which are strict autotrophs, nitrite oxidizers are capable of heterotrophic growth under some limited conditions. Even anaerobic heterotrophic growth may be possible, with nitrite is much slower than other heterotrophic bacteria and slower than when nitrite oxidizers grow autotrophically.

### Heterotrophic Nitrification

Several heterotrophic microorganisms oxidize either ammonium or organic nitrogen to nitrite or nitrate. Heterotrophic nitrifiers include both fungi (e.g., *Aspergillus*) and bacteria (e.g., *Alcaligenes*, *Artbrobacter* spp., and some actinomycetes). A particularly interesting bacterium is *Thiosphaera pantotropha*, which is a heterotrophic nitrifier that can also denitrify under aerobic conditions. Unlike the autotrophic nitrifiers, heterotrophic nitrifiers gain no energy through this activity. In fact, it is uncertain what benefit heterotrophic nitrifiers gain by oxidizing organic

nitrogen, although hydroxamic acids, which act as **siderophores**, compounds involved in iron acquisition, are one type of oxidized nitrogen product.

The relative importance of heterotrophic versus autotrophic nitrification is still debated. In pure cultures, the highest rates of nitrite or nitrate production are just one-tenth that of autotrophic nitrifiers, which would suggest that heterotrophic nitrifiers are of minor importance. The case is not as clear-cut in soils.

### Factors Affecting Nitrification in the Environment

Many interesting factors control nitrification in soils. The decision tree shown in Figure 2 is one way of assessing the relative importance of these factors. The most important, or most commonly limiting, factors are listed at the top of the decision tree. If all factors are favorable, then nitrification is possible; if any factor is unfavorable, then significant rates of nitrification are unlikely. Implied by this organization is that the factors affecting nitrification rates are multiplicative (i.e., they interact). The dashed line shows that alleviating a limiting factor has the potential to increase the growth of nitrifiers, hence increasing their populations in soil.

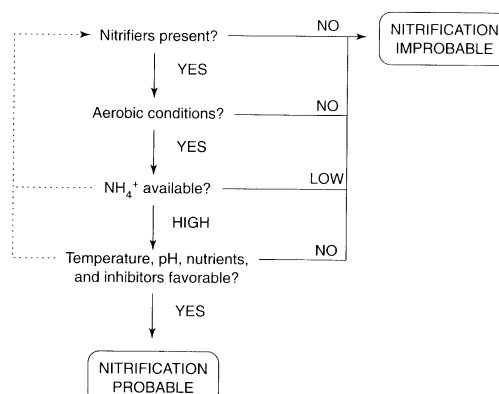


Figure 2: Factors regulating nitrification in soil

**Nitrifier population:** For nitrification to occur, either autotrophic or heterotrophic nitrifiers must be present. Nitrifiers are present in most soils; however, they may be present in populations too low to be of much importance in producing nitrate. For example, if we extrapolate from the activity of pure cultures of autotrophic nitrifying bacteria, we can calculate that about  $3 \times 10^5$  nitrifiers  $\text{g}^{-1}$  soil are required for nitrification rate of  $1 \text{ mg N kg}^{-1} \text{ day}^{-1}$ . Unfertilized soils contain far fewer nitrifiers than this, often  $10^3$  to  $10^4 \text{ g}^{-1}$ , but upon nitrogen fertilization, nitrifier populations have been observed to increase to more than  $10^6 \text{ g}^{-1}$ . A similar response is often seen when wildland soils are disturbed. In their natural state, many wildland soils have very low concentrations of nitrate and small populations of nitrifiers. If disturbance increases the availability of ammonium, nitrifier populations and rates of nitrification often increase gradually until they reach a new higher steady state.

**Soil aeration:** Because nitrifiers are almost exclusively aerobic microorganisms, soil must have sufficiently high concentrations or fluxes of oxygen for nitrification to occur. Like general aerobic heterotrophic activity, nitrification is typically optimal when bulk soils are near field capacity or at about 60% water-filled pore space. It should be noted, however, that even flooded soils and sediments normally have a narrow aerobic zone where nitrification occurs. As oxygen becomes more limiting, autotrophic nitrifiers produce relatively more nitric oxide and nitrous oxide.

**Substrate availability:** Provided that aerobic conditions exist, the most important regulating factor for nitrification is substrate availability, particularly ammonium availability. Studies have shown that ammonium and nitrite oxidation follow Michaelis-Menten kinetics. The saturation constants for these oxidations are in the same range as typical soil concentrations of ammonium and nitrite, which suggests that substrate availability is often limiting to growth and activity. This is consistent with the previous description of nitrifier populations being limited by available substrate.

Because nitrification activity is often dominated by autotrophic nitrifiers, it is possible that carbon dioxide concentrations may also influence the growth of nitrifiers. The higher carbon dioxide concentration found in soils compared to the atmosphere may be beneficial to nitrifying bacteria, as long as oxygen does not become limiting. Carbonate equilibrium may also help to poise the soil pH at a level more favorable for nitrifiers.

**Soil pH:** Nitrification rates are often low in soils below pH 4.5, particularly in agricultural soils. At high pH, nitrite can accumulate because of greater inhibition of nitrite oxidizers relative to ammonia oxidizers. These observations, along with the fact that most isolates of autotrophic nitrifying bacteria grow best at neutral pH, support the generalization that autotrophic nitrifiers are neutrophilic. Nevertheless, high rates of nitrification or high concentrations of nitrate have been observed in many acid ( $\text{pH} < 4.5$ ) soils. Several explanations for this apparent paradox include acidophilic autotrophic nitrifiers, heterotrophic nitrifiers, and alkaline microsites.

Perhaps the most common explanation given for nitrate production in acid soils is the presence of heterotrophic nitrifiers.

**Miscellaneous soil and environmental factors:** Clearly factors such as temperature, water potential, salinity, and availability of nutrients other than nitrogen all have the potential to affect the activity of nitrifiers. Because of their slow growth rate and relatively inefficient metabolism, nitrifiers are thought to be more sensitive to temperature, particularly low temperatures, than

common heterotrophs. Psychrophilic nitrifiers have been identified, however. There have also been indications that phosphorus availability may limit nitrification rates in some soils. Some scientists have suggested that nitrification is a sensitive indicator of alterations in the soil environment.

### **Fate of Nitrate in the Soil Environment**

Like ammonium, nitrate has many competing fates in the soil ecosystem. Because it is an anion, nitrate is easily leached. Removal of nitrate from the soil by leaching has several consequences. Obviously, nitrate leaching represents a loss of available nitrate from the plant-soil system. When nitrate is leached, it must be accompanied by an equivalent amount of cations to maintain charge balance. Thus soils are also depleted of cations when nitrate is leached. The leaching of basic cations, such as  $K^+$  and  $Ca^{2+}$  reduces the base saturation of a soil and increases exchangeable acidity. Nitrate that leaches eventually enters ground and surface waters, where it may have potentially adverse environmental effects. High concentrations of nitrate in surface water can lead to **eutrophication** (the sudden enrichment of natural water with excess nutrients which can lead to the development of algal blooms and other vegetation). High concentration of nitrate are associated with methemoglobinemia (blue-baby syndrome), which is now quite rare. A further environmental hazard may be the production of carcinogenic nitrosamines from reactions between nitrite and secondary amines.

### **Assimilatory Nitrate Reduction**

Nitrate can be assimilated by plants and microorganisms. The process of assimilatory nitrate reduction requires energy for the conversion of nitrate to ammonium and subsequent incorporation of ammonium into amino acids. Consequently, this process is regulated by nitrogen availability, and nitrate utilization is expected when energy is in excess relative to the concentrations of ammonium or organic-nitrogen compounds. For this reason, soil scientists believed assimilation of nitrate (also called *nitrate immobilization*, a term which emphasizes that the nitrogen has been made unavailable to the other organism) by soil microorganisms to be minor. However, there is growing evidence that nitrate immobilization is an important process in some soils.

Plants vary in their ability and preference for ammonium and nitrate uptake. When both ammonium and nitrate are equally available in soil solution, it is energetically more favorable for plants to use ammonium because nitrate must be reduced prior to use by the plant. In many cases, however, plants are not energy limited, so reducing power is available to convert nitrate to

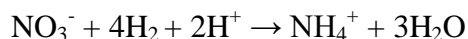
ammonium. This is particularly true for plants that reduce nitrate in leaf tissue, where this reduction is coupled to light energy and photosynthesis. The relative energy cost of ammonium versus nitrate metabolism is even more difficult to calculate in a heterogeneous medium like soil, being a more extensive root system to access the less mobile ammonium.

### **Dissimilatory Nitrate Reduction**

Nitrate can also be reduced by dissimilatory process. In acidic soils of pH 5 or less, nitrogen gases can be produced chemically, with NO formation from the dismutation of nitrate being the major reaction. Nitrate can also react with the amino groups of organic-nitrogen compounds to form dinitrogen. These *chemodenitrification* reactions are typically minor compared to biological dissimilatory processes.

In most soils, respiratory denitrification is usually the major dissimilatory process that reduce nitrate. In *nonrespiratory denitrification*, organisms produce nitrous oxide under aerobic conditions but do not gain energy from this reaction. Non-respiratory Denitrification is accomplished by a wide range of bacteria, fungi, and algae; it has even been associated with higher plants and animals, although in these latter cases nitrous oxide is probably produced by associated microorganisms. With the exception of a few genera (e.g., *Propionibacterium*, *Lactobacillus*, and *Fusarium*), the fraction of nitrate converted to nitrous oxide is generally less than 25%. The importance of non-respiratory Denitrification in converting nitrate into nitrous oxide in nature is currently unknown, primarily because of the difficulty of distinguishing this process from others that produce nitrous oxide.

Nitrate-respiring bacteria convert nitrate to nitrite under anaerobic conditions. In doing so, they gain energy via oxidative phosphorylation ( $161 \text{ kJ}$  or  $38 \text{ kcal mol}^{-1} \text{ NO}_3^-$ ). The enteric bacteria, which are facultative anaerobes, are typical examples; however, many of these can also further reduce nitrite to ammonium. Complete reduction of nitrate to ammonium is known as **dissimilatory nitrate reduction to ammonium**, or DNRA. Under anaerobic conditions, several genera of bacteria are capable of DNRA. The overall reaction for DNRA is:



A total of  $8e^-$  are transferred during this reduction, with an energy yield of  $600 \text{ kJ}$  ( $143 \text{ kcal}$ )  $\text{mol}^{-1} \text{ NO}_3^-$ , or  $150 \text{ kJ}$  ( $36 \text{ kcal}$ )  $\text{mol}^{-1} 2e^-$  transferred. The first step in this reaction is the conversion of nitrate to nitrite, which is linked to energy production via oxidative phosphorylation. Most bacteria that carry out DNRA do not gain any additional energy from the subsequent reduction of nitrite to ammonium.



## Denitrification

The major form of dissimilatory nitrate reduction in soil is respiratory denitrification, more commonly known simply as **denitrification**. This refers to the reduction of nitrate to gaseous nitrogen products, principally dinitrogen and nitrous oxide, coupled to energy production via oxidative phosphorylation. It is an example of anaerobic respiration, where an alternate electron acceptor other than oxygen is used. The overall stoichiometry of the reaction is:



Denitrification gains slightly less energy per mole of  $\text{NO}_3^-$  than DNRA (560 kJ or 134 kcal); however, it gains more per mole of  $2e^-$  transferred (224 kJ or 53 kcal). The higher thermodynamic yield per  $2e^-$  is consistent with the observation that denitrification is likely to be the most important reductive process in soils where heterotrophic organisms are often limited by available carbon.

Denitrification bacteria comprise 0.1 to 5% of the total bacteria population of soils and represent a wide range of taxonomic groups. Despite this diversity, soil denitrifiers are dominated by members of the genus *Pseudomonas*, with species of *Alcaligenes*, *Flavobacterium*, and *Bacillus* also common. Thus aerobic heterotrophs predominate, although autotrophic denitrifiers are also known. Furthermore, bacteria normally associated with other nitrogen transformations (e.g., *Azospirillum*, *Nitrosomonas*, and *Rhizobium*) denitrify under certain conditions. Because denitrification is described as an anaerobic process carried out by prokaryotes, it is interesting to note the recent isolation of an aerobic denitrifying bacterium (*Thiosphaera pantotropa*) and of fungi that appear to have the capability for respiratory denitrification.

## Denitrification Enzymes

The denitrification pathway involves four reductive steps and their corresponding enzymes. Dissimilatory nitrate reductase (Nar) is a membrane-bound enzyme that contains molybdenum/iron, and labile sulfur groups. It catalyzes the reduction of nitrate to nitrite, with the generation of ATP. This step is common to all organisms that dissimilate nitrate. Synthesis of Nar is inhibited by oxygen, as is the activity of existing enzyme.

The reduction of nitrite to nitric oxide is a defining characteristic of denitrifiers. This step is catalyzed by nitrite reductase (Nir). The presence of nitrate induces the expression of Nir. We now know that nitric oxide is an obligate intermediate in the denitrification pathway, and it is converted to nitrous oxide by the activity of nitric oxide reductase (Nor). Synthesis of Nar is repressed by oxygen and induced by nitrogen oxides.

The final enzyme of the denitrification pathway is nitrous oxide reductase (Nos), which reduces nitrous oxide to dinitrogen. Like other denitrification enzymes, synthesis of Nos is regulated by oxygen and nitrogen oxides. Its activity is more strongly inhibited by oxygen, and is more sensitive to low pH than the other enzymes. Thus, under high oxygen or low pH conditions, relatively more nitrous oxide than dinitrogen is produced. Nitrous oxide reductase is also strongly inhibited by sulfide and acetylene. This latter characteristic is the basis for the so-called **acetylene block method**, which greatly spurred the study of denitrification in natural environments. In the presence of acetylene, nitrous oxide accumulates and can be measured with a gas chromatograph to quantify rates of denitrification.

### **Factors Affecting Denitrification in the Environment**

As with nitrification, the regulation of denitrification can be thought of as hierarchy from more to less important factors. The presence of denitrifiers is seldom a limitation. Denitrifiers make up a reasonable large fraction of the soil bacteria, probably because most of them normally exist as aerobic heterotrophs that switch to nitrate as an alternate electron acceptor when oxygen becomes unavailable. Therefore, in most soils the formation of anaerobic conditions is the most important controlling factor, followed in order by the availability of nitrate and carbon. Of course, there are always instances when other factors, such as temperature and soil pH, may be extreme enough to limit denitrification.

**Soil aeration:** Although examples of aerobic denitrification have been observed fairly recently, denitrification in soils and other natural habitats is predominantly an anaerobic process. Oxygen affects denitrification by regulating enzyme synthesis and by inhibiting enzyme activity. Enzyme synthesis is less sensitive to oxygen than is activity. The differential sensitivity of denitrifier enzyme activity explains why the ratio of nitrous oxide to dinitrogen increases as oxygen concentrations increase.

Once anaerobic conditions are established, denitrification rates are most often limited by either nitrate or carbon availability. Which of these two is more limiting depends on their relative abundance, which is often related to soil type, plant community, or management practices.

**Nitrate availability:** In most natural soil systems, nitrate, the alternate electron acceptor, is more limiting than carbon even if heterotrophic microorganisms are carbon-limited under aerobic conditions. There are several reasons for this. First, the obligatory aerobic heterotrophs, which make up the bulk of the microbial biomass, can no longer compete for carbon in the absence of oxygen. In effect, carbon is less limiting under anaerobic conditions. Second, in many wild land

soils, such as forest soils, grasslands, and natural wetlands, net nitrogen mineralization and net production of nitrate are small. Habitats that are anaerobic or experience long periods of anaerobiosis such as some sediments and anaerobic digestors, are similarly limited in nitrate availability because nitrification is inhibited under anaerobic conditions. Finally, even in soils with relatively high rates of nitrification, there are many competing fates for the nitrate produced. Many of these fates, including leaching and DNRA, are enhanced under wet, anaerobic soil conditions.

***Carbon availability:*** Researchers often find correlations between measures of carbon availability (e.g., respiration rates) and denitrification rates. This is evidence that denitrification rates are influenced by carbon availability; however, it is confounded because carbon utilization also influences oxygen supply. Nevertheless, some controlled studies have shown a positive response of denitrification to additions of carbon under anaerobic conditions. It is likely that carbon limitation is greatest in soils with high nitrification rates or large nitrate pools, such as fertilized agricultural soils.

***Miscellaneous soil and environmental factors affecting denitrification:***

Denitrification responds to temperature as do most biological processes, increasing as temperature increases until a maximum is reached, above which activity declines rapidly. Mesophilic denitrifiers predominate in most soils, although activity has been measured near freezing and also under thermophilic conditions. Temperature is likely to have a more complex effect on denitrification than on some other soil process because it also affects oxygen and nitrous oxide solubility, gas diffusion coefficients, and the oxygen consumption activity of other heterotrophs.

The response of denitrifiers to pH is similar to that of other soil heterotrophs, which usually function best near neutrality. Biological denitrification has been measured in some acidic soils, but rates are usually low and the measurements can be potentially confounded by chemodenitrification. Relatively little research has been focused on denitrification in soils of high pH.

## **NITRIFICATION INHIBITORS**

Slow-release fertilizers such as sulfur-coated urea are used to limit nitrification in soil because they limit the rate at which  $\text{NH}_4^+$  becomes available to nitrifiers. There are also many specific inhibitors of nitrification that differ in their mode of action. Chelating agents remove the Cu cofactor in ammonium monooxygenase (AMO). Some examples of chelating agents are:

Hydrazinecarbothiamide	$\text{NH}_2\text{CSNNH}_2$
Diethyldithiocarbamate	$(\text{C}_2\text{H}_5)_2\text{NS}_2\text{Na}$
Allylthiourea	$\text{CH}_2=\text{CHCH}_2\text{NHCSNH}_2$
Thiourea	$\text{H}_2\text{NCSNH}_2$

Other chemicals also chelate Cu in ammonia monooxygenase and interfere with electron transport by cytochromes. Some examples are sodium azide ( $\text{NaN}_3$ ) and potassium cyanide ( $\text{KCN}$ ).

Pyridine compounds interact with the active site of ammonia monooxygenase. Examples include pyridine, nitrapyrin, and picolinic acid. The chemical nitrification inhibitor with the greatest commercial application in agriculture is nitrapyrin (2-Chloro-6 [trichloromethyl] pyridine), which is sold under the brand name “N-Serve”.

Acetylene gas ( $\text{C}_2\text{H}_2$ ) at very low concentrations (1-10Pa) inhibits nitrification. It is a very useful inhibitor for laboratory studies of nitrification in soil because heterotrophic nitrification is unaffected by acetylene at similar concentrations.

Virtually all of these chemical inhibitors prevent nitrification at the level of  $\text{NH}_4^+$  oxidation. This makes perfect sense because if they inhibit  $\text{NO}_2^-$  oxidation, several bad things will happen. First,  $\text{NO}_2^-$  is still an anion so it tends to leach whenever it rains. Second,  $\text{NO}_2^-$  is much more toxic to plant growth than is either  $\text{NH}_4^+$  or  $\text{NO}_3^-$ , so plant toxicity occurs if  $\text{NO}_2^-$  accumulates. Third,  $\text{NO}_2^-$  in groundwater might rise to concentration high enough so that it poses a problem to adults drinking the water. Fourth,  $\text{NO}_2^-$  is more reactive than is either  $\text{NH}_4^+$  or  $\text{NO}_3^-$ , so it has a greater tendency to break down into gaseous products that affect the global atmosphere.