CHAPTER 14

Transformations of Nitrogen

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Nature works only in cycles, there are no straight lines.
The forward movement is provided by time. Everything within it must revolve.

—Anonymous

Perhaps more time and effort have been invested in studying the nitrogen (N) 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 (Vitousek et al. 1997). Current concerns include high concentrations of nitrate in ground and surface waters and the contribution of gaseous nitrogen oxides, such as NO and N_2O, to large-scale environmental problems of acid rain, ozone depletion, and greenhouse warming (Chapter 19). 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 intent of this chapter is to present the generally accepted workings of the nitrogen cycle and its associated organisms. Such generalizations inherently leave out some of the details and deviations from the accepted norm. For those who desire more of the nuances of the nitrogen cycle, a few exceptions to the rules are presented in the chapter's box material.

THE NITROGEN CYCLE

An overview of the nitrogen cycle is presented in Figure 14–1. Nitrogen is present in various forms (Table 14–1)—primarily as dinitrogen gas (N_2), organic nitrogen (in plants, animals, microbial biomass, and soil organic matter), and ammonium (NH_4^+) and nitrate (NO_3^-) ions. Microbially mediated processes transform nitrogen from one

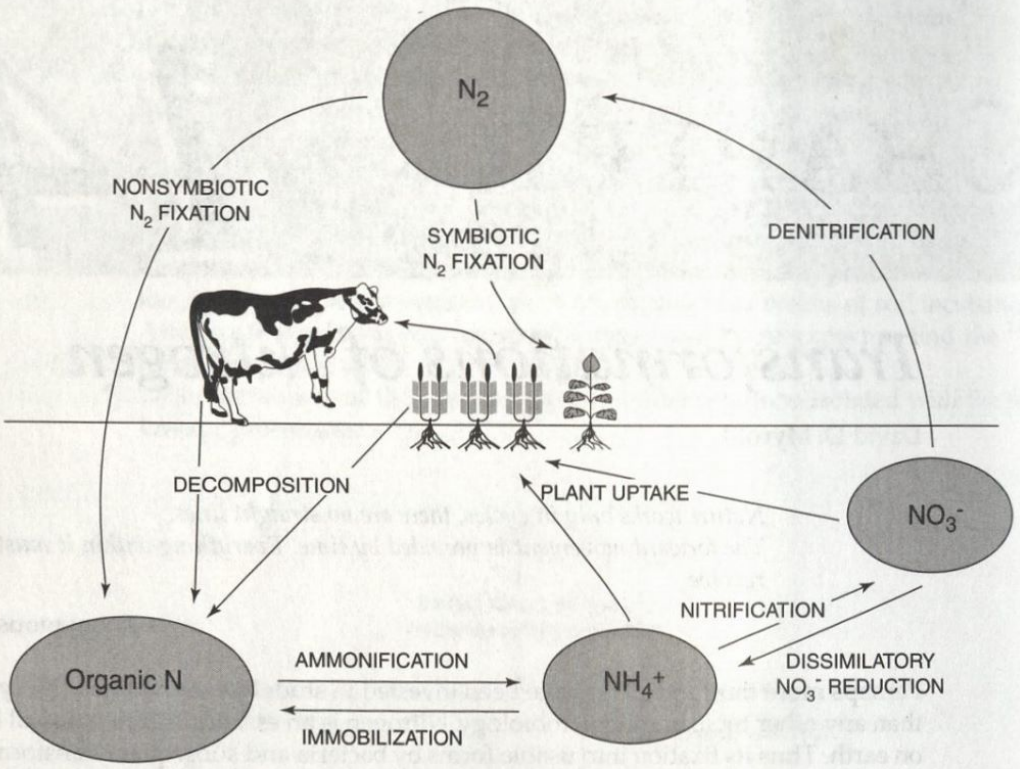


FIGURE 14–1
Overview of the nitrogen cycle, showing major pools (shaded areas) and transformations (lines) of N.

TABLE 14–1 Pool Sizes of Terrestrial Nitrogen Based on Soil to a Depth of One Meter

Pool	Typical Size (Range) (g N m^{-2})	Remarks
N_2 (Dinitrogen)	1,150 (230–27,500)	Minimum based on 0.25 m^3 air-filled pore space in the soil; maximum based on soil air plus a 30-m tall cylinder of air above the soil surface (e.g., a tall forest stand).
Organic N	725 (100–3,000)	From Post et al. (1985); typical value is median of reported soil N contents. Histosols are not included and would likely contain 3,000–8,000 g N m^{-2}.
Plant N	25 (1–240)	Minimum based on desert regions, maximum based on agricultural crops (Olson and Kurtz, 1982) and forest systems (Waring and Schlesinger, 1985; Anderson and Spencer, 1991).
NH_4^+ (Ammonium)	1 (0.1–10)	Assumes 1 m^3 soil at a bulk density of 1.25 Mg m^{-3} and typical NH_4^+ concentrations for soil extracts.
NO_3^- (Nitrate)	5 (0.1–30)	Assumes 1 m^3 soil at a bulk density of 1.25 Mg m^{-3} and typical NO_3^- concentrations for soil extracts.

form to another. Certain bacteria can transform dinitrogen to ammonia (NH_3) by a process known as dinitrogen fixation. The processes 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.

The nitrogen cycle can be divided into three subcycles, nested within each other (Fig. 14–2):

- *Elemental*: emphasizing the biological oxidation-reduction reactions that interconvert nitrogen and dinitrogen into various chemical forms,
- *Autotrophic:* driven by plant nitrogen uptake, which is fueled by photosynthesis and converts inorganic nitrogen (NH_4^+ and NO_3^-) into organic, nitrogen-containing plant constituents,
- *Heterotrophic*: linked to decomposition processes and driven by the need of heterotrophic organisms for preformed carbon (C).

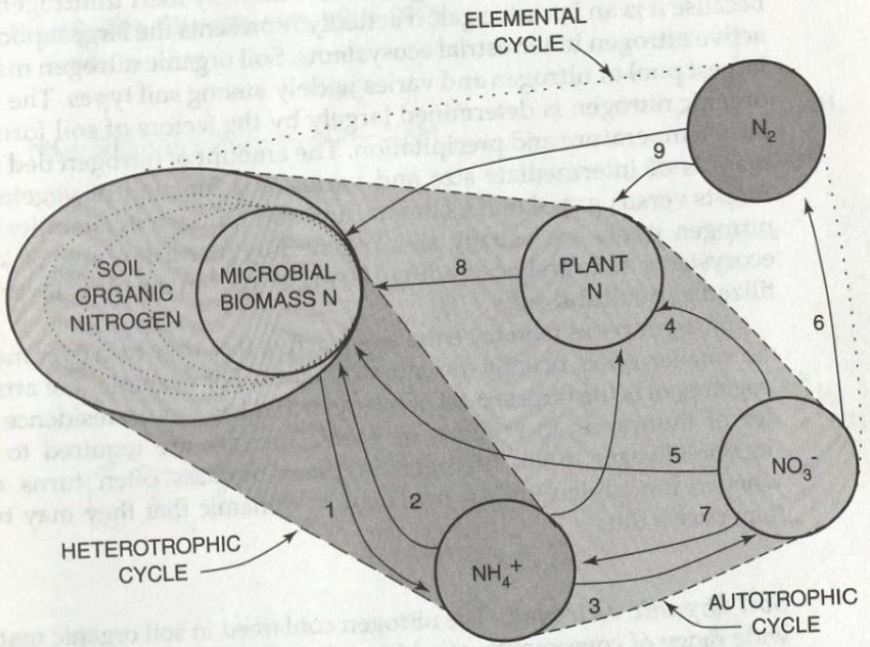


FIGURE 14–2
Detailed nitrogen cycle showing major processes and control points. The nitrogen cycle consists of three overlapping subcycles. The demand of heterotrophic organisms for organic carbon drives one subcycle, the heterotrophic cycle (long dashed lines), where ammonium is both consumed and produced. The demand of plants for inorganic nitrogen drives the second subcycle, the autotrophic cycle (short dashed lines). Finally, the oxidation and reduction of nitrogen by microorganisms drives the third subcycle, the elemental cycle (dotted lines), where nitrogen is converted into various forms. All the subcycles contain the soil organic-nitrogen and ammonium pools. The soil organic-nitrogen pool contains several nitrogen fractions of differing biological availability as well as a separate microbial biomass nitrogen pool. Important biological transformations of nitrogen include: (1) ammonification; (2) immobilization; (3) autotrophic nitrification; (4) plant uptake; (5) nitrate immobilization; (6) denitrification; (7) dissimilatory nitrate reduction to ammonium; (8), decomposition; and (9) N_2 fixation. *Based on Jansson and Persson (1982). Used with permission.*

These three subcycles function in concert. Generally, the heterotrophic cycle turns over most quickly, followed by the autotrophic cycle, with the elemental cycle being the slowest. This general pattern results because the three subcycles are in competition for one or more of the pools of nitrogen and the outcome of this competition determines which subcycle dominates. There are two main control points of this competition: the ammonium and the nitrate pools, as discussed later in this chapter. First, however, we need to review the various forms of nitrogen and their characteristics, because these important fundamentals influence the microbial transformations of nitrogen in soil.

Forms of Nitrogen

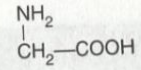
The sizes of the nitrogen pools vary over several orders of magnitude (Table 14–1). Although we tend to ignore the relatively inert dinitrogen pool, probably because it is an invisible gas, it actually represents the largest pool of biologically active nitrogen in terrestrial ecosystems. Soil organic 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 (e.g., forests versus grasslands), climate, and soil nitrogen availability. Soil inorganic-nitrogen pools are usually small, generally just a few mg N kg^{-1} in natural ecosystems and rarely exceeding 100 mg N kg^{-1} in the plow layer of recently fertilized agricultural soils.

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.

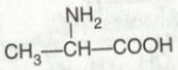
Soil Organic Nitrogen. The nitrogen contained in soil organic matter occurs in a wide range of compounds, of which only about half can be definitively identified. Naturally occurring organic-nitrogen compounds isolated from soils include proteins and amino acids, microbial cell-wall polymers and amino sugars, nucleic acids, and a variety of vitamins, antibiotics, and metabolic intermediates (Figs. 14–3 and 14–4). 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 (Table 14–2). It is interesting to note that the range given for amino-sugar nitrogen, which is found mainly in microbial cell walls (Fig. 14–4), is similar to that often found for microbial biomass nitrogen, which is typically about 5% of total soil nitrogen.

a. Common Amino Acids

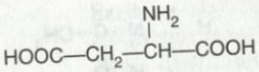
Glycine



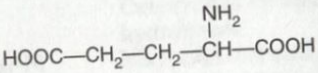
Alanine



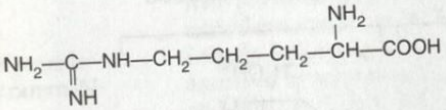
Aspartic acid



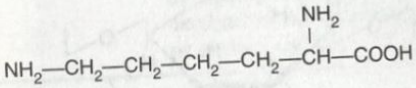
Glutamic acid



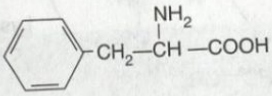
Arginine



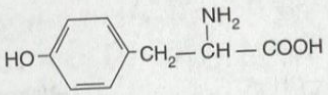
Lysine



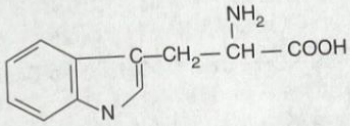
Phenylalanine



Tyrosine

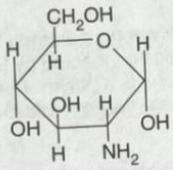


Tryptophan

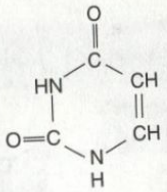


b. Amino Sugar

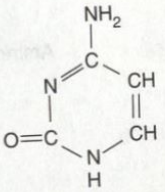
Glucosamine



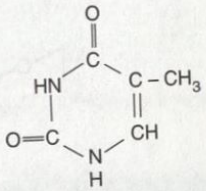
c. Nucleic Acids



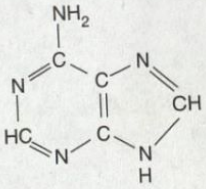
Uracil



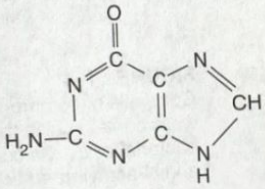
Cytosine



Thymine



Adenine



Guanine

FIGURE 14–3
Examples of important organic-nitrogen compounds in soil: (a) common amino acids, (b) an amino sugar, and (c) common nucleic acids.

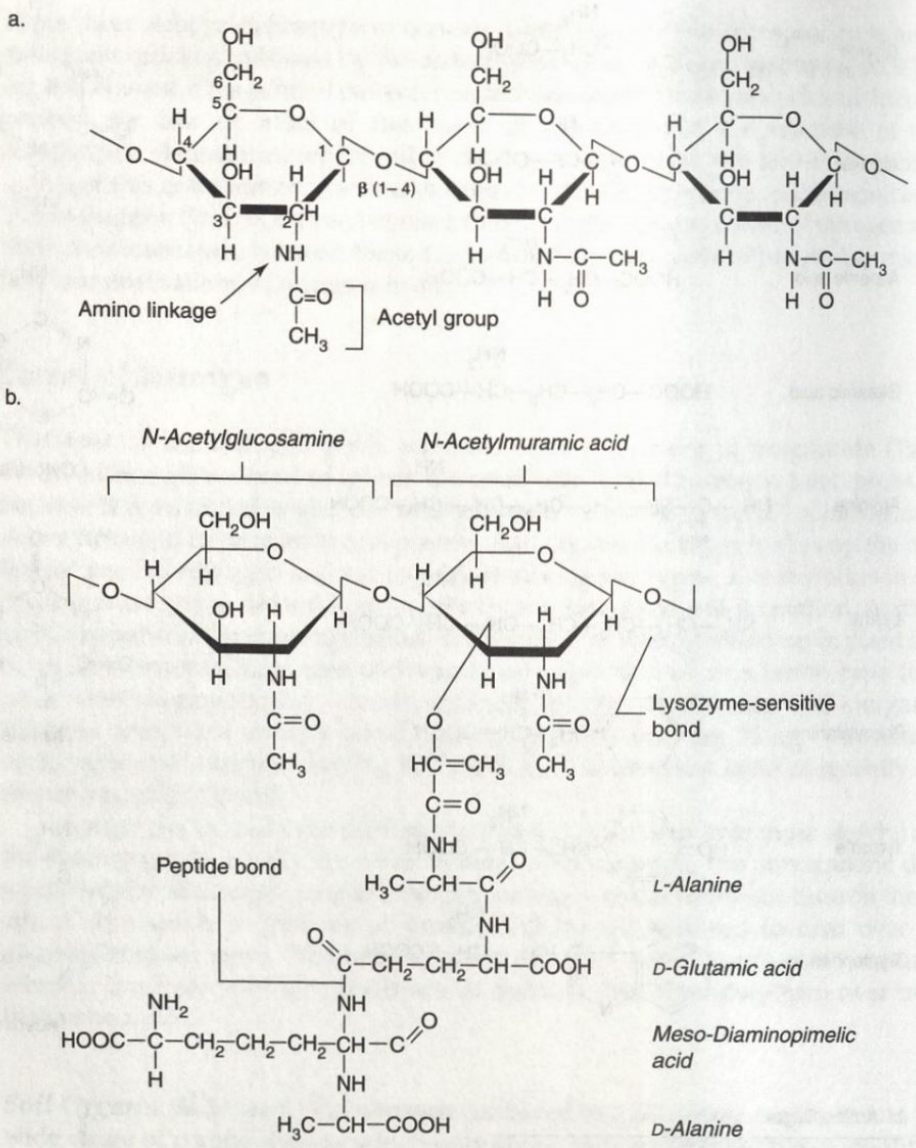


FIGURE 14–4
Cell walls of common soil microorganisms are composed of polymers of amino sugars: (a) fungal cell walls contain chitin that consists of N-acetylglucosamine moieties connected by β1–4 linkages and (b) bacterial cell walls contain a peptidoglycan layer whose backbone is a polymer of N-acetylglucosamine and N-acetylmuramic acid connected by β1–4 linkages that has an oligopeptide side chain.

One purpose of soil organic-matter fractionation schemes is to determine which fractions are most active in nutrient turnover (Box 14–1). One method of measuring turnover is to add inorganic nitrogen labeled with ^{15}N (a stable, heavy isotope) and measure how it is partitioned among the various organic-nitrogen fractions. Such studies of recently immobilized nitrogen have shown that the amino acid and unknown hydrolyzable nitrogen fractions are often relatively

TABLE 14–2 Classic Fractionation of Soil Nitrogen Based on Acid Hydrolysis

Form of Nitrogen	Definition and Method	Typical Range (% of Soil N)
Acid insoluble-N	Largely aromatic N. Nitrogen remaining in soil residue following acid hydrolysis (6 *M* HCl).	10–20
Ammonia-N	Exchangeable NH_4^+ plus amide N. Ammonia recovered from hydrolysate by steam distillation with MgO.	20–35
Amino acid-N	Protein, peptide, and free amino acid N. Determined by ninhydrin reaction of hydrolysate.	30–45
Amino sugar-N	Microbial cell walls. Ammonia recovered from hydrolysate by steam distillation with phosphate-borate buffer at pH 11.2 minus the ammonia-N fraction.	5–10
Hydrolyzable unknown-N	Largely unknown but contains non-α-amino-N of arginine, tryptophan, lysine, and proline. The hydrolyzable N not accounted for as ammonia, amino acids, or amino sugars.	10–20

Based on Stevenson (1982).

BOX 14–1

Exception to the Rule 1: Inorganic Nitrogen Is the Only Form of Nitrogen Important for Plant and Microbial Uptake

Inorganic nitrogen fertilizers are the most widely used tool to manage crop nitrogen fertility, particularly in intensively managed agricultural and forested production systems. Even with organic-nitrogen amendments, such as manure, it is generally assumed that the organic nitrogen must first be converted into inorganic nitrogen before plants can utilize it. Microorganisms are also thought to use organic-nitrogen compounds as a source of carbon rather than of nitrogen. Recent work, however, suggests that organic-nitrogen compounds may be used directly as a source of nitrogen for soil microorganisms and for plants (Näsholm et al., 1998), particularly plants with roots colonized by certain types of mycorrhizal fungi (Chapter 12). Much of the most readily available organic nitrogen is likely found in soluble, small organic molecules, which make up the dissolved organic-nitrogen (DON) pool. Although the size of the DON pool is typically smaller than the inorganic nitrogen in the soil solution of agricultural soils, DON can be the dominant form of soluble nitrogen in soils receiving low inputs of nitrogen. Studies in unpolluted forest ecosystems show that DON can be the major form of nitrogen lost to ground and surface waters (Perakis and Hedin, 2002). As a result of these recent findings, more attention is being paid to DON and its role in the nitrogen cycle.

enriched in ^{15}N whereas the acid insoluble fraction shows very little incorporation of the labeled 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 forms of nitrogen and their characteristics are shown in Table 14–3.

TABLE 14–3 Important Inorganic Nitrogen Compounds Found or Produced in Soil

Compound	Formula	Oxidation State	Form in Soil	Major Attributes
Ammonium	NH_4^+	−3	Fixed in clay lattice, dissolved, as gaseous ammonia (NH_3)	Cationic, rather immobile, volatilizes as NH_3 at high pH, assimilated by plants and microbes, substrate for autotrophic nitrification (NH_3 oxidation)
Hydroxylamine	NH_2OH	−1	Not detected	Intermediate in NH_3 oxidation
Dinitrogen	N_2	0	Gas	Largest pool of N, relatively insoluble, substrate for N_2 fixation, end product of denitrification
Nitrous oxide	N_2O	+1	Gas, dissolved	Greenhouse gas and implicated in ozone destruction, very soluble, an intermediate in denitrification, by-product of nitrification
Nitric oxide	NO	+2	Gas	Chemically reactive, an intermediate in denitrification, by-product of nitrification
Nitrite	NO_2^-	+3	Dissolved	Normally present at very low concentrations, toxic, product of NH_3 oxidation, substrate for NO_2^- oxidation, an intermediate in denitrification
Nitrate	NO_3^-	+5	Dissolved	Anionic, mobile, readily leached, assimilated by plants and microbes, end product of nitrification, substrate for denitrification

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.

Extracellular Enzymes Important in Nitrogen Transformations. The major extracellular enzymes produced by microorganisms depolymerize proteins, aminopolysaccharides (microbial cell walls), and nucleic acids and hydrolyze urea (Table 14–4).

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. These 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 (Fig. 14–4), 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

TABLE 14–4 Extracellular Enzymes Involved in Microbial Nitrogen Mineralization

Substrates	Enzymes	Products
Proteins	Proteinases, proteases	Peptides, amino acids
Peptides	Peptidases	Amino acids
Chitin	Chitinase	Chitobiose
Chitobiose	Chitobiase	N-acetylglucosamine
Peptidoglycan	Lysozyme	N-acetylglucosamine and N-acetylmuramic acid
DNA and RNA	Endonucleases and exonucleases	Nucleotides
Urea	Urease	NH_3 and CO_2

Based on Ladd and Jackson (1982).

by chitobiase. This process is analogous to the enzymatic degradation of cellulose (Chapter 13). Several enzymes work to degrade the peptidoglycan portion of bacterial cell walls. Lysozyme is perhaps the most well known. It breaks the β 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.

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.

Considerable research has focused on the interactions between extracellular enzymes and soil mineral and organic constituents. These interactions are complex. For example, both the enzyme, which is a protein, and the substrate may be adsorbed onto clay surfaces. This may act to stabilize the enzyme or substrate and protect it from degradation. This type of stabilization provides one explanation for the presence of free DNA in soils. If the active conformation of an extracellular enzyme is altered by adsorption, this will likely inactivate the enzyme, but if the catalytic site is not affected, the enzyme may remain active. In the latter case, the protected, active enzyme may be an important catalyst as long as its substrate is accessible.

Intracellular Enzymes Important in Nitrogen Transformations. 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 (NH_2-CR_3) and amide (NH_2-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 cofactor 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 phosphate (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.

Immobilization (Assimilation)

Microbes and other organisms assimilate ammonium by two primary pathways (Fig. 14–5): glutamate dehydrogenase and glutamine synthetase-glutamate synthase (GOGAT). When ammonium is present in relatively high concentrations (> 0.1 m*M* or about 0.5 mg N kg^{-1} soil), glutamate dehydrogenase, acting with $NADPH_2$ as a coenzyme, can add ammonium to α-ketoglutarate to form glutamate.

In most soils, ammonium is present at low concentrations, which results in low intracellular ammonium concentrations. Under these conditions, the second ammonium assimilation system is operable. The GOGAT pathway is complex. The first step requires ATP to add ammonium to glutamate to form glutamine. The second step transfers the ammonium from glutamine to α-ketoglutarate to form two glutamates. Once ammonium has been incorporated into glutamate, it can then be transferred to other carbon skeletons by various transaminase reactions to form additional amino acids.

Net Ammonium Production or Consumption

Several factors influence whether there is net production or consumption of ammonium by microorganisms in soil. The general principle is that net immobilization of ammonium occurs if the availability of nitrogen is limiting; otherwise, net production occurs. In most soils, the growth and activity of heterotrophic microorganisms are limited primarily by the amount of available carbon; net nitrogen mineralization is the norm in these soils. This is actually not surprising, because plants, which require inorganic nitrogen, grow well in most soils.

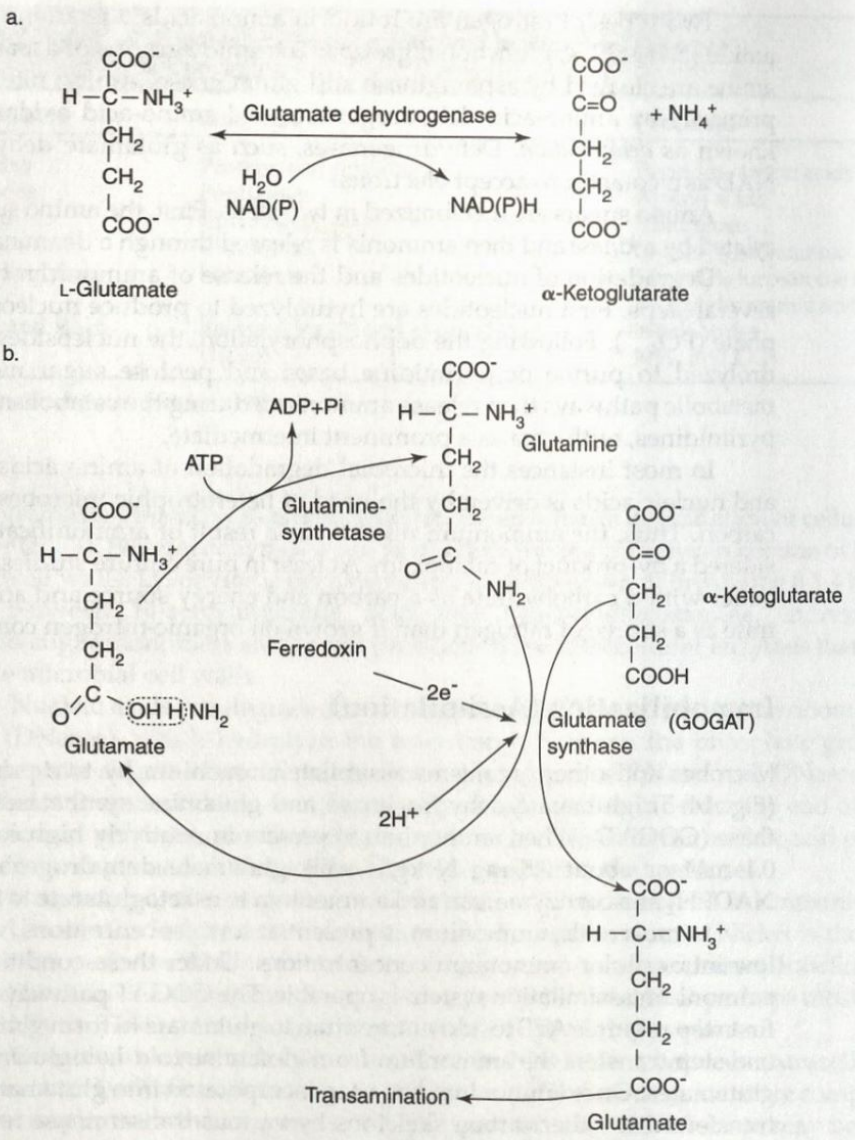


FIGURE 14–5
Two main pathways of ammonium assimilation: (a) glutamate dehydrogenase, which is a reversible reaction that works at higher ammonium concentrations and (b) glutamine synthase-glutamate synthetase (GOGAT), which is induced and functions at low ammonium concentrations. This uptake system requires energy.

When plant residues are returned to the soil or organic amendments are added, we can predict what effect this addition has on nitrogen availability. Adding a material high in carbon, such as sawdust, will likely immobilize inorganic nitrogen, whereas adding a material relatively high in nitrogen, such as manure, will provide available nitrogen. Most organic materials are about 45% carbon

by mass; consequently, the ratio of carbon to nitrogen is determined largely by the concentration of nitrogen in the material. Decades of research have shown that when organic amendments with **C/N ratios** below 20/1 are added to soils, net ammonium production results; at wider C/N ratios, inorganic nitrogen from the soil is immobilized. This critical, or "break-even," C/N ratio can be calculated from basic principles based on knowledge of the C/N ratio of the heterotrophic microorganisms and the **yield coefficient**, the amount of substrate carbon converted to microbial biomass (Box 14–2).

The C/N ratio of soil microorganisms ranges from 4 or 5 for bacteria to as high as 15 for fungi. Given that fungal biomass is often about twice that of bacteria in many soils, a typical C/N ratio for soil microbial biomass is about 8. Yield coefficients vary widely, depending on the type of organic matter (substrate quality),

BOX 14–2

Calculating the Critical C/N Ratio That Determines Whether Nitrogen Is Mineralized or Immobilized

Start with these basics:

- Fungi typically make up about two-thirds of the total microbial biomass; bacteria make up about one-third.
- Fungi typically convert about 44% of the carbon of readily decomposable organic matter into cell biomass; thus their yield coefficient (Y) is 0.44.
- Bacteria typically convert about 32% of the carbon of readily decomposable organic matter into cell biomass; thus their yield coefficient (Y) is 0.32.
- Fungal cells commonly have a C/N ratio of about 10.
- Bacterial cells commonly have a C/N ratio of about 4.

We can calculate that the decomposing organic substrate must have a C/N ratio of 20 or less in order to meet the nitrogen needs of the microbial decomposers by the following steps:

Step 1. Calculate the average microbial yield coefficients and C/N ratios;

$$Y = (2/3)0.44 + (1/3)0.32 = 0.4$$
$$C/N = (2/3)10 + (1/3)4 = 8$$

Step 2. Calculate how much microbial biomass carbon is produced;
100 g substrate C → 60 g CO_2-C + 40 g microbial biomass C

Step 3. Calculate how much microbial biomass nitrogen is produced;
40 g microbial biomass C ÷ C/N ratio of 8 = 5 g microbial biomass N

Step 4. Calculate the substrate C/N ratio that would be needed;
substrate C/N ratio = 100 g substrate C ÷ 5 g substrate N = 20

type of microorganism, and environmental conditions. Readily degradable organic compounds, such as simple sugars, may have yield coefficients as high as 0.6, whereas assimilation efficiencies for complex, recalcitrant compounds, such as lignin, may be less than 0.1. A reasonable average yield coefficient for plant-derived substrates is about 0.4. Fungi are typically more efficient than bacteria and thus have higher yield coefficients, perhaps 0.5 versus 0.4. Environmental conditions that stress microorganisms would generally decrease the yield coefficient because more energy is required for cell maintenance than for growth. Another example of the effect of environmental factors is that yield coefficients are often much lower under anaerobic conditions, primarily because anaerobic metabolism normally produces less energy per mole of substrate.

Net production of ammonium is influenced not only by environmental factors and the C/N ratio of substrates and microbes but also by other biotic factors. Most important of these is the role that soil animals play as predators of the primary decomposers, bacteria and fungi. About 30% of the yearly net nitrogen mineralization is directly released by the activities of soil animals, such as protozoa and nematodes. When soil animals prey upon microorganisms, ammonium is often released as a waste product because the predators have a C/N ratio similar to their prey, which results in an excess of nitrogen due to loss of carbon as carbon dioxide during metabolism. This phenomenon may be important in the rhizosphere where "grazing" by soil protozoa facilitates liberation of available nitrogen for plant growth (Chapter 8).

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 quinones, 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 chemoautotrophic nitrification is ammonia oxidation, the conversion of ammonium (actually, ammonia at the enzyme level) to nitrite (NO_2^-) by the ammonia-oxidizing bacteria of the "Nitroso-" genera (Table 14–5). Nitrite is then oxidized to nitrate by the nitrite-oxidizing bacteria of the "Nitro-" genera.

In addition to the oxidations by the autotrophic nitrifying bacteria, other microbes can produce nitrite and nitrate by enzymatic oxidation processes that

TABLE 14–5 Chemoautotrophic Nitrifying Bacteria

Class	Genus	Species	Physiological Traits	Habitats
NH_3 oxidizers				
Betaproteobacteria	*Nitrosomonas*	*europeae*	Halotolerant	Sewage treatment, eutrophic freshwater, brackish water
		eutrophus		Soil
		halophila		Eutrophic freshwater
		communis	Urease	Oligotrophic freshwater, soil
		nitrosa	Urease	Marine environment
		oligotropha		
		ureae		
		aestuarii	Halophilic, urease	
		marina		
	Nitrosospira	*briensis*	Some have urease	Soil, rocks, freshwater
		multiformis		
		tenuis		
Gammaproteobacteria	*Nitrosococcus*	*nitrosus*	Halophilic, some have urease	Marine environment
		oceani		
NO_2^- oxidizers				
Alphaproteobacteria	*Nitrobacter*	*alkalicus*	Halotolerant	Soda lakes
		hamburgensis		Freshwater, soil, rocks
		vulgaris		
		winogradskyi		
Gammaproteobacteria	*Nitrococcus*	*mobilis*	Halophilic	Marine environment
Deltaproteobacteria	*Nitrospina*	*gracilis*	Halophilic	Marine environment
Nitrospira	*Nitrospira*	*marina*	Halophilic	Marine environment
		moscoviensis		Freshwater

Based on Koops and Pommerening-Röser (2001).

are not linked to microbial growth. For example, the many genera of methane-oxidizing bacteria contain a membrane-bound methane monooxygenase enzyme that will oxidize ammonia as well as methane, which is an interesting linkage between carbon and nitrogen cycling. Perhaps more widespread is heterotrophic nitrification discussed in the following section, the oxidation of ammonium or organic-nitrogen compounds by a variety of heterotrophic bacteria and fungi.

Ammonia Oxidation

Ammonia-oxidizing bacteria are thought to be a relatively defined and coherent group. Recent phylogenetic work based on 16S rRNA sequences has largely confirmed this: except for two strains of *Nitrosococcus oceanus*, all autotrophic ammonia oxidizers are tightly clustered together phylogenetically within the Betaproteobacteria. Representatives from each of the three genera of ammonia oxidizers have been isolated from soil. Although *Nitrosomonas* has been the best characterized and most studied ammonia oxidizer, particularly with respect to

its enzymology and the biochemistry of ammonia oxidation, *Nitrosospira* is thought to be the dominant ammonia oxidizer in many soils.

The overall reaction for the conversion of ammonia to nitrite is:

$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H^+ + H_2O$$

This oxidation is a $6e^-$ transfer that yields 271 kJ (65 kcal) mol^{-1} NH_3. The first step in the reaction is the conversion of NH_3 to NH_2OH (hydroxylamine) by the membrane-bound *ammonia monooxygenase* enzyme:

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$$

This reaction is endergonic and requires a small amount of energy. It is not coupled to ATP synthesis. Like many monooxygenase enzymes, ammonia monooxygenase has broad substrate specificity. It can oxidize methane but does so at much lower rates than methane-oxidizing bacteria. Ammonia monooxygenase has been shown to **cometabolize** several other small organic compounds, including some halogenated organics such as trichloroethylene, chlorinated ethanes, and chloroform. A practical characteristic of the broad substrate specificity of ammonia monooxygenase is that it will bind irreversibly to acetylene. Thus, acetylene is a useful inhibitor of ammonia oxidation. Several other inhibitors of ammonia oxidation have been developed and used in agriculture (Box 14–3).

Hydroxylamine is converted through several undefined steps to nitrite with an overall reaction of:

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$

This is an energy-yielding reaction, with two of the electrons produced passing down the electron-transport chain to oxygen (O_2) 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 (HNO) is thought to be produced from the oxidation of NH_2OH and may be the source of some of the nitric oxide (NO) that is released as a by-product of nitrification. The final step(s) in the production of nitrite are not well-defined.

Two other products of ammonia oxidation are nitrous oxide (N_2O) and acidity. Ammonia oxidizers contain a nitrite reductase, which is capable of reducing NO_2^- to N_2O. 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 soils by releasing one mole of 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.

BOX 14–3

Nitrification Inhibitors

For several decades scientists have attempted to find specific inhibitors of ammonia oxidation with the ultimate goal of commercializing these compounds for use in agriculture. The initial motivation was to increase the efficiency of fertilizer nitrogen (typically ammonium compounds or urea) use by crop plants to maximize economic yield. More recently the goal has expanded to include minimizing environmental consequences of nitrate in excess of plant needs. Some of the more successful or commercially available nitrification inhibitors are listed in the following table.

Effectiveness of nitrification inhibitors expressed as average percent inhibition of nitrification in three soils treated with 5 mg active ingredient kg^{-1} soil that had been amended with 200 mg $(NH_4)_2SO_4$-N kg^{-1} soil and incubated at 25°C.

Common name(s)	*Chemical*	*Inhibition (%)* 14 d	28 d
	2-Ethynylpyridine	97	87
	Phenylacetylene*	92	55
Dwell	Etridiazole	90	75
N-serve, nitrapyrin	2-Chloro-6-(trichloromethyl)pyridine	85	65
ATC	4-Amino-1,2,4-triazole	87	60
	2,4-Diamino-6-trichloromethyl triazine	76	41
DCD, dicyan	Dicyandiamide	61	15
AM	2-Amino-4-chloro-6-methylpyrimidine	60	37
ST	Sulfathiazole	52	17
Tu	Thiourea	2	0

*Average of two soils after 10 and 30 days incubation.
Data from McCarty and Bremner (1986, 1990).

Currently the most widely used nitrification inhibitors are probably N-serve and DCD, although Dwell, a more recent product, would seem to be a more effective choice. The acetylenic compounds 2-ethynylpyridine and phenylacetylene also seem to show promise, along with wax-coated calcium carbide. As the wax coat surrounding the calcium carbide granules breaks down, the calcium carbide reacts with water to form acetylene, a potent inhibitor of ammonia oxidation. Field studies with irrigated cotton and flooded rice have shown these acetylenic compounds to be at least as good as N-serve in increasing fertilizer nitrogen recovery (Freney et al., 1993; Keerthisinghe, Freney, and Mosier, 1993).

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, with the following stoichiometry:

$$NO_2^- + 1/2O_2 \rightarrow NO_3^-$$

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

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

This reaction yields 77 kJ (18 kcal) mol^{-1} nitrite utilized, about one-third that of ammonia oxidation. Nitrite oxidation can be competitively inhibited by chlorate (ClO_4^-), which is useful in experimental studies to distinguish between 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 oxidoreductase reducing nitrate to nitrite. Heterotrophic growth by nitrite oxidizers 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, Arthrobacter* spp., and some actinomycetes). A particularly interesting bacterium is *Thiosphaera pantotropha (Paracoccus pantotrophus)*, 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, however, where the relative rates of the two nitrification processes have been assessed with inhibitors (e.g., nitrapyrin and acetylene are thought to only block autotrophic nitrification) or the use of ^{15}N. For example, in one study most of the nitrate produced in a coniferous forest soil was from heterotrophic nitrification.

Factors Affecting Nitrification in the Environment

Many interacting factors control nitrification in soils. The decision tree shown in Figure 14–6 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.

Nitrifier Populations. 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 3×10^5 nitrifiers g^{-1} soil are required for a nitrification rate of 1 mg N kg^{-1} day^{-1} (Schmidt, 1982). Unfertilized soils contain far fewer nitrifiers than this, often 10^3 to 10^4 g^{-1}, but upon nitrogen fertilization, nitrifier populations have been observed to increase to more than 10^6 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

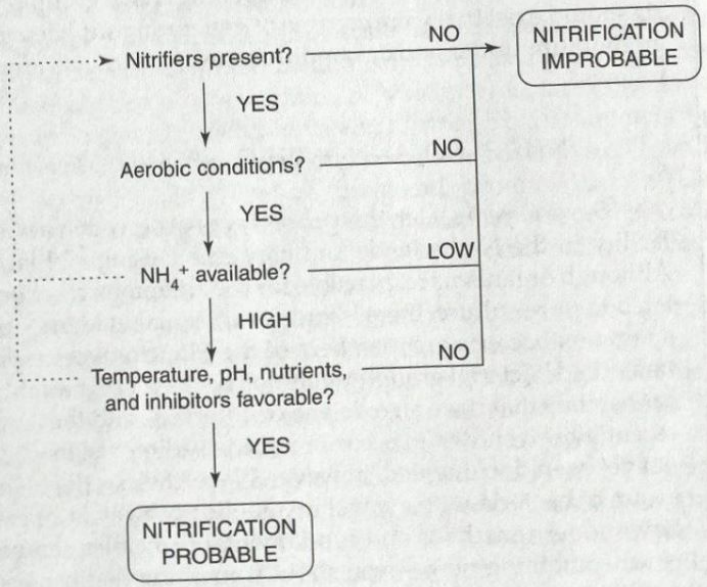


FIGURE 14–6
Hierarchy of factors regulating nitrification in soil. The dashed lines suggest that these factors may limit nitrifier populations.

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 (Box 14–4), soils 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 of a few millimeters where nitrification occurs. As oxygen becomes more limiting, autotrophic nitrifiers produce relatively more nitric oxide and nitrous oxide.

BOX 14–4

Exception to the Rule 2: Anaerobic Ammonia Oxidation

The previous discussion of nitrification suggests that it is a strictly aerobic process. Although this is likely the predominant case in most soils, there is an exception. The story of anaerobic ammonia oxidation, or *anammox*, is a wonderful tale of discovery, which illustrates the importance of understanding the basic principles of microbial metabolism.

About 25 years ago, it was postulated, based on thermodynamic considerations of redox reactions (Chapter 3), that a group of **lithotrophic** bacteria should exist that were capable of generating sufficient energy by oxidizing ammonium under anoxic conditions using nitrite as an electron acceptor as follows.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O;\ -358\ kJ\ (86\ kcal)\ mol^{-1}\ NH_4^+$$

Several years later, this process was observed in a wastewater treatment facility in the Netherlands and confirmed using ^{15}N labeling experiments. Although organisms responsible for the anammox reaction have not been isolated in pure culture, there is strong molecular evidence to suggest that some anammox bacteria are members of the Planctomycetes phylum, a relatively unstudied bacterial group. Anammox bacteria are thought to be well adapted for habitats that have an oxic-anoxic interface, and the anammox process has recently been shown to occur in marine sediments. Its occurrence in soil has not yet been documented, however. Nevertheless, the identification of a new group of bacteria with a novel metabolic pathway is an excellent example of how modern methods and fundamental principles can be combined to discover something new—even about something that has been as well studied as the nitrogen cycle.

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 (Chapter 10). 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 autotrophic nitrifiers often dominate nitrification activity, 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 ($pH < 4.5$) soils. Several explanations for this apparent paradox include acidophilic autotrophic nitrifiers, heterotrophic nitrifiers, and alkaline microsites (De Boer and Kowalchuk, 2001).

Several ammonia-oxidizing and nitrite-oxidizing bacteria have been isolated from low pH soils, with *Nitrosospira* and *Nitrobacter* being the most common genera. With the exception of a few strains of *Nitrobacter* isolated from acid forest soil (Hankinson and Schmidt, 1988), however, nitrifier isolates are neutrophilic or alkalophilic in pure culture. More recently co-cultures of *Nitrosospira* and *Nitrobacter* have been isolated that hydrolyze urea and produce nitrate (De Boer and Laanbroek, 1989). Adding urea stimulates nitrification above the small increase in pH associated with urea hydrolysis. It appears that acidophilic nitrifiers may exist, or at least operate, as consortia in acid soils.

Perhaps the most common explanation given for nitrate production in acid soils is the presence of heterotrophic nitrifiers. As mentioned previously, studies with either inhibitors that are thought to be specific for autotrophic nitrifiers or $^{15}NH_4^+$ have shown significant heterotrophic nitrification in some acid soils. Most isolated heterotrophic nitrifiers are not acidophilic, however.

In a habitat as diverse as soil, it would not be surprising to find microsites of higher pH. These might be associated with surfaces of minerals, organic matter, or even roots; however, conclusive evidence is lacking and empirical evidence is mixed. It is perhaps more likely that microsites are created by the activity of microorganisms themselves. For example, ammonia released during mineralization of organic nitrogen by heterotrophs or from urea hydrolysis may alter microsite pH. Such an effect

would be difficult to distinguish from enhanced substrate availability, however. Thus we see that the puzzle of nitrification in acid soils remains to be solved.

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.

Allelochemical Inhibitors. The observation of low nitrate concentrations in soils of climax communities in natural ecosystems led to a theory of specific inhibition of nitrification by allelochemicals produced by the climax vegetation. This theory suggests that tannins and polyphenols are among the more important allelochemical agents. Subsequent research has demonstrated that this allelochemical theory is probably not the major reason for the low soil nitrate concentrations. Active competition for nitrate by plant uptake and microbial immobilization are probably dominant (Box 14–5). In fact, several studies that examined a wide suite of proposed allelochemicals have failed to show any direct effect on nitrification rate or nitrifier populations. That is not to say that naturally occurring inhibitors of nitrification do not occur, rather that such compounds do not seem to be the primary reason for low soil nitrate concentrations in climax communities.

BOX 14–5

Exception to the Rule 3: Nitrification in Soils of Mature Forests

Observations of nitrate pool sizes and net production of nitrate during incubations of undisturbed forest soils led to the suggestion that nitrate is a relatively unimportant pool of available nitrogen in these soils and that nitrification was not an important process. Various reasons were given for this, including low populations of autotrophic nitrifiers, limited substrate (ammonium) availability, and allelochemical inhibition. When mature forests were disturbed by clear-cutting, for example, a subsequent increase in soil and streamwater nitrate concentrations and net nitrification rates often resulted. This change was in part explained by a reduced amount of plant competition for ammonium. Collectively, these observations led to the dogma that as ecosystems mature, losses of nitrogen are reduced and nitrogen cycling becomes more conservative, primarily because nitrification is turned off.

BOX 14–5 *(continued)*

Work using ^{15}N isotope dilution methods to measure the gross rates of ammonification and immobilization and nitrification and nitrate immobilization has revised our view of how nitrogen is conserved in mature forest ecosystems. An excellent example is the study by Davidson, Hart and Firestone (1992), who applied these methods to forest soils from an old-growth (more than 100 years) mixed-conifer forest and a 10-year-old mixed-conifer plantation in northern California and found the following:

*Nitrogen cycling characteristic**	*Young forest*	*Old-growth forest*
Inorganic N (mg N m^{-2})†		
NH_4^+	340	210
NO_3^-	300	80
N mineralization (mg N m^{-2} d^{-1})‡		
Net	6.8	2.6
Gross	90	280
N nitrification (mg N m^{-2} d^{-1})‡		
Net	6.6	−0.4
Gross	67	45

*Data are for top 9 cm of mineral soil.
†Mean of 7 dates from November through September.
‡Mean of 3 dates from November through April.

In agreement with past studies, the researchers found higher inorganic-nitrogen concentrations and greater net nitrogen mineralization and net nitrification in soil from the young, recently disturbed stand. However, gross rates of inorganic-nitrogen production were 10 to 100 times greater than net rates, with high rates of nitrification (gross nitrate production) being nearly as high in the soil from the mature forest as from the young stand. Thus, significant nitrification occurred in soils of both stands but immobilization rates of ammonium and nitrate were relatively higher compared to net production rates in the older stand. Probably a more significant point drawn from these data was how rapidly both the ammonium and nitrate pools turned over, with mean residence times on the order of days or hours and with greater turnover in the old-growth stands. This work agrees with the dogma that as forests age, soil nitrogen cycling becomes more conservative, not because nitrification is lessened but because nitrate immobilization is greater and not because turnover slows down, because it may actually increase. Nitrogen seems to be conserved because of enhanced immobilization, which is likely fueled by greater carbon availability (Hart, et al., 1994).

FATE OF NITRATE IN THE SOIL ENVIRONMENT

Like ammonium, nitrate has many competing fates in the soil ecosystem (Fig. 14–1). 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 waters can lead to **eutrophication** (the sudden enrichment of natural waters with excess nutrients which can lead to the development of algal blooms and other vegetation). Current federal regulations require that drinking water contain < 10 mg NO_3^--N L^{-1} (read as "mg nitrate nitrogen per liter"). Note that this level is similar to the World Health Organization (WHO) standard of 50 mg NO_3^- L^{-1}. High concentrations 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

Plants and microorganisms can assimilate nitrate. 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 other organisms) by soil microorganisms to be minor. However, there is growing evidence that nitrate immobilization is an important process in some soils (Box 14–5).

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, because it may be more efficient for a plant to use nitrate than to put energy into growing a more extensive root system to access the less mobile ammonium.

Dissimilatory Nitrate Reduction

Nitrate can also be reduced by dissimilatory processes (Table 14–6). In acidic soils of pH 5 or less, nitrogen gases can be produced chemically, with NO formation from the dismutation of nitrite being the major reaction. Nitrite can also react with the amino groups of organic-nitrogen compounds to form dinitrogen by the van Slyke reaction. These *chemodenitrification* reactions are typically minor compared to biological dissimilatory processes.

In most soils, respiratory denitrification is usually the major dissimilatory process that reduces nitrate. In *nonrespiratory denitrification*, organisms produce nitrous oxide under aerobic conditions but do not gain energy from this reaction. Nonrespiratory 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 associated microorganisms probably produce nitrous oxide. 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 nonrespiratory 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 kJ or 38 kcal mol^{-1} 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

TABLE 14–6 Processes That Reduce Nitrate

Process	Products	Energy Conserved	Regulated By	Soil Condition Where Expected
Assimilatory NO_3^- assimilation*	NH_4^+	no	NH_4^+, organic N	low NH_4^+ concentration
Dissimilatory				
Chemodenitrification	NO>>N_2, N_2O	no		acidic
Nonrespiratory denitrification	N_2O	no	?	aerobic
NO_3^- respiration†	NO_2^-	yes	O_2	anaerobic
Dissimilatory NO_3^- reduction to NH_4^+	NH_4^+>>N_2O	a few strains	O_2	anaerobic
Respiratory denitrification	N_2>N_2O>NO	yes	O_2	anaerobic

*Also known as NO_3^- immobilization.
†All known organisms that dissimilate NO_3^- to NH_4^+ are also NO_3^- respirers, but most NO_3^- respirers accumulate NO_2^-.
Based on Tiedje (1994).

conditions, several genera of bacteria are capable of DNRA (Table 14–7). The overall reaction for DNRA is:

$$NO_3^- + 4H_2 + 2H^+ \rightarrow NH_4^+ + 3H_2O$$

A total of $8e^-$ are transferred during this reduction, with an energy yield of 600 kJ (143 kcal) mol^{-1} NO_3^-, or 150 kJ (36 kcal) 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 as it is with the nitrate respirers. Most bacteria that carry out DNRA do not gain any additional energy from the subsequent reduction of nitrite to ammonium. Some species of *Campylobacter*, *Desulfovibrio*, and *Wolinella* are exceptions that do apparently generate ATP from this final reduction step. Because most DNRA bacteria gain only minimal energy from this reduction, some researchers have suggested that this process serves to either detoxify the nitrite intermediate or to regenerate reducing equivalents through the reoxidation of NADH. The latter process seems to be the most important because:

- Under conditions of energy (carbon) limitation, nitrite accumulates in the medium, which suggests that nitrite may not be particularly toxic and that energy is not produced by further reduction to ammonium.

TABLE 14–7 Bacteria That Can Dissimilate Nitrate to Ammonium (DNRA)

Genus	Typical Habitat
Obligate anaerobes	
Clostridium	Soil, sediment
Desulfovibrio	Sediment
Selenomonas	Rumen
Veillonella	Intestinal tract
Wolinella	Rumen
Facultative anaerobes	
Citrobacter	Soil, wastewater
Enterobacter	Soil, wastewater
Erwinia	Soil
Escherichia	Soil, wastewater
Klebsiella	Soil, wastewater
Photobacterium	Seawater
Salmonella	Sewage
Serratia	
Vibrio	Sediment
Microaerophile	
Campylobacter	Oral cavity
Aerobes	
Bacillus	Soil, food
Neisseria	Mucous membranes
Pseudomonas	Soil, water

Based on Tiedje (1988).

- Under conditions of excess carbon, ammonium is the major product, presumably because of the need to regenerate NAD.

These observations in pure culture agree with ecological studies that find DNRA bacteria to predominate over respiratory denitrifiers in carbon-rich environments, such as sediments and sewage sludge, whereas denitrifiers predominate in more carbon-poor habitats, such as soils.

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:

$$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O$$

Denitrification gains slightly less energy per mole of 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.

Denitrifying bacteria comprise 0.1% to 5% of the total bacterial population of soils and represent a wide range of taxonomic groups (Table 14–8). This taxonomic diversity spans phylogenetic 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 (*Paracoccus pantotrophus*) (Robertson and Kuenen, 1984) 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 (Fig. 14–7). 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.

TABLE 14–8 Genera of Denitrifying Bacteria

Genus	Interesting Characteristics of Some Species
Organotrophs	
Alcaligenes	Commonly isolated from soils
Agrobacterium	Some species are plant pathogens
Aquaspirillum	Some are magnetotactic, oligotrophic
Azospirillum	Associative N_2 fixer, fermentative
Bacillus	Spore former, fermentative, some species thermophilic
Blastobacter	Budding bacterium, phylogenetically related to *Rhizobium*
Bradyrhizobium	Symbiotic N_2 fixer with legumes, e.g., soybean
Branhamella	Animal pathogen
Chromobacterium	Purple pigmentation
Cytophaga	Gliding bacterium; cellulose decomposer
Flavobacterium	Common soil bacterium
Flexibacter	Gliding bacterium
Halobacterium	Halophilic
Hyphomicrobium	Grows on one-C substrates, oligotrophic
Kingella	Animal pathogen
Neisseria	Animal pathogen
Paracoccus	Halophilic, also lithotrophic
Propionibacterium	Fermentative
Pseudomonas	Commonly isolated from soil, very diverse genus
Rhizobium	Symbiotic N_2 fixer with legumes, e.g., alfalfa, clover
Wolinella	Animal pathogen
Phototrophs	
Rhodopseudomonas	Anaerobic, reduce SO_4^{2-}
Lithotrophs	
Alcaligenes	Use H_2, also heterotrophic, commonly isolated from soil
Bradyrhizobium	Use H_2, also heterotrophic, symbiotic N_2 fixer with legumes
Nitrosomonas	NH_3 oxidizer
Paracoccus	Use H_2, also heterotrophic, halophilic
Pseudomonas	Use H_2, also heterotrophic, commonly isolated from soil
Thiobacillus	S oxidizer
Thiosmicrospira	S oxidizer

Based on Firestone (1982) and Tiedje (1988, 1994).

The reduction of nitrite to nitric oxide is a defining characteristic of denitrifiers. This step is catalyzed by nitrite reductase (Nir). Two forms of Nir are known: one contains copper (Cu-Nir), and the other contains cytochromes *c* and d_1 (heme-Nir). Heme d_1 is the active site of the heme-Nir. About two-thirds of the denitrifiers contain heme-Nir, including most of the *Pseudomonas* strains, *Alcaligenes*, *Paracoccus*, *Thiobacillus*, and *Azospirillum*. Copper is required for activity of Cu-Nir, which is less common than heme-Nir but more widespread taxonomically. It is found in some *Pseudomonas* and *Alcaligenes* strains, *Bacillus*, *Rhizobium*, *Nitrosomonas*, and others. Researchers have not determined whether the two types of Nir are related to performance of the denitrifiers in the environment although differences in their distribution have been observed (Priemé, Braker, and Tiedje,

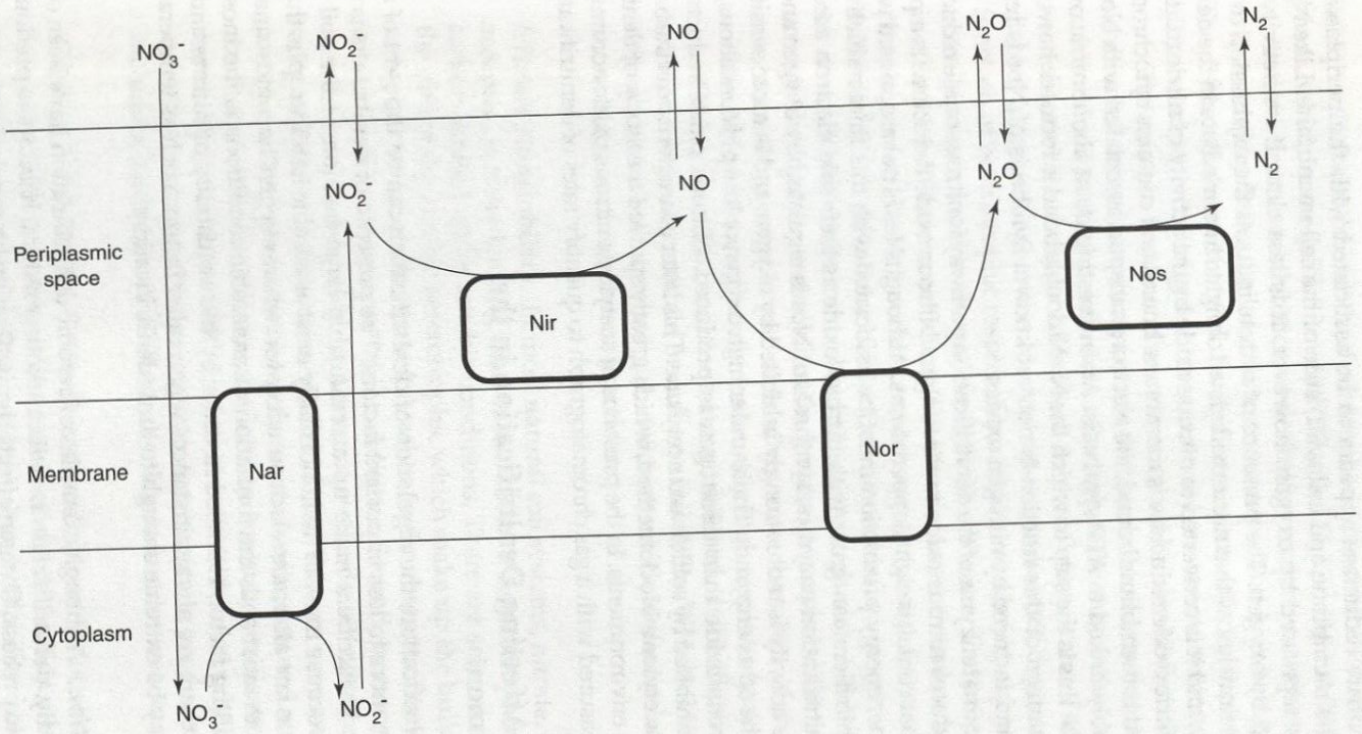


FIGURE 14–7
Denitrification pathway showing the location of the four denitrification enzymes: Nar—nitrate reductase, Nir—nitrite reductase, Nor—nitric, Averill, and Tiedje oxide reductase, Nos—nitrous oxide reductase. *Adapted from Ye, Averill, and Tiedje. (1994). Used with permission.*

2002). Nitrite reductase appears to be associated with the periplasmic (i.e., between the membrane and cell wall) side of the cell membrane. Like Nar, Nir synthesis is repressed by oxygen; however, it is less clear if its activity is directly inhibited by oxygen. 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). Nitric oxide reductase is membrane bound and contains cytochromes *b* and *c*. Because it is membrane bound, the electron transport associated with Nor activity is presumably linked to ATP synthesis. An interesting point about nitric oxide reduction is that this is the step in which the N=N double bond is formed; however, the exact mechanism of this reaction is not yet known. Synthesis of Nor 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. Nitrous oxide reductase is a periplasmic protein that contains eight copper atoms. Although Nos is not associated with the cell membrane, energy production must be associated with this final reductive step because denitrifiers can grow with nitrous oxide as their sole electron acceptor. 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 a hierarchy from more to less important factors. The presence of denitrifiers is seldom a limitation. Denitrifiers make up a reasonably 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. Synthesis of Nar and Nir is derepressed when oxygen concentra-

tions reach about one-tenth of atmospheric concentrations (2 kPa O_2 in the gas phase, which is in equilibrium with 29 μmol O_2 L^{-1} H_2O at 20°C). As oxygen concentrations decrease, inhibition of denitrifier enzyme activities is relieved sequentially, with Nar being the least oxygen sensitive and Nos being the most sensitive. The differential sensitivity of denitrifier enzyme activity explains why the ratio of nitrous oxide to dinitrogen increases as oxygen concentrations increase.

The oxygen concentration experienced by denitrifiers in soil is a complex function of many interacting factors that control soil aeration. Aeration in soils occurs predominantly by diffusion, although there may be some transport by convection or even of oxygen dissolved in percolating water. Diffusion of oxygen is directly proportional to the concentration gradient of oxygen, which is largely a function of heterotrophic respiratory activity consuming oxygen in the soil and inversely proportional to the path-length for diffusion. The proportionality constant is called the diffusion coefficient (D). It varies over several orders of magnitude depending on soil texture, water content, and tortuosity. For example, D varies from 0.208 cm^2 s^{-1} in air to 2.6×10^{-5} cm^2 s^{-1} in water (i.e., oxygen diffuses through water about 10,000 times more slowly than through air). A detailed example will be covered later as a case study, but the general principle is that rates of denitrification are generally greatest in wet soils when more than 80% of the pore space is filled with water and where there is reasonably high respiratory activity (Box 14–6).

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 obligately 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 wildland soils, such as forest soils, grasslands, and natural wetlands, net nitrogen mineralization and net production of nitrate are small. Habitats that are very 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.

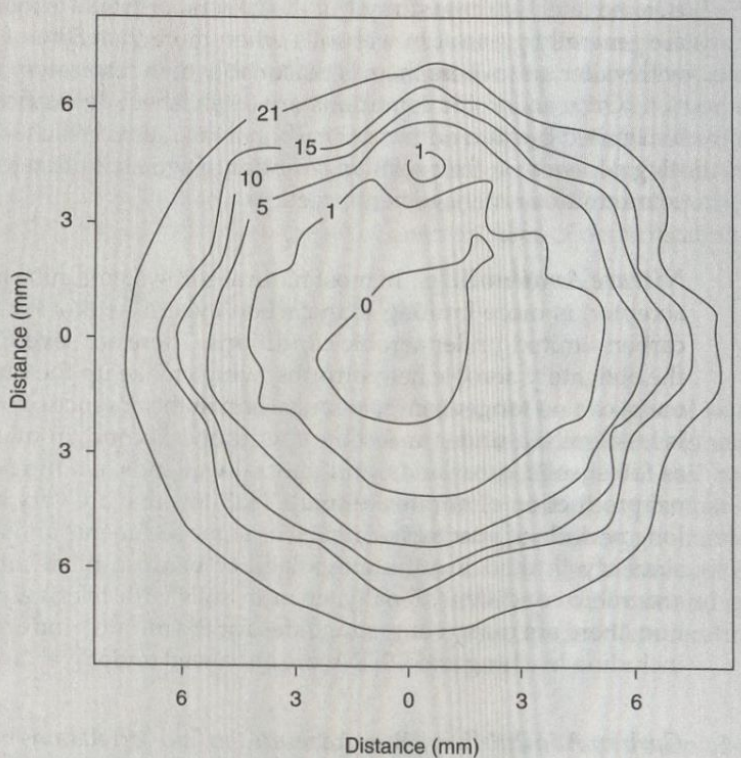
Carbon Availability. Researchers often find correlations between measures of carbon availability (e.g., respiration rates) and denitrification rates. This is evidence that denitrification rates are influenced by carbon availability; however, it is confounded because carbon utilization also influences oxygen supply. Nevertheless, some controlled studies have shown a positive response of denitrification to additions of car-

BOX 14–6

Anaerobic Microsites

An intriguing puzzle about denitrification is how this anaerobic process can occur in aerobic soils with nearly atmospheric concentrations of oxygen. Although at least one aerobic denitrifier is now known, most of the evidence points to the establishment of anaerobic zones within an otherwise aerobic soil profile. The most studied and best documented of these anaerobic microsites occur within large soil aggregates.

Using several simplifying assumptions and typical values for diffusion and consumption, it is possible to calculate how large a soil aggregate must be for an anaerobic microsite to develop (Currie, 1961; Smith, 1980). This calculation can be extended to estimate the anaerobic volume of soils using aggregate size distributions and to relate the anaerobic volume to denitrification rates.



Oxygen contours in a saturated soil aggregate as measured with a microelectrode. Numbers on contour lines are volume percent of oxygen.
Adapted from Sexstone et al. (1985b).

BOX 14–6 *(continued)*

The most direct demonstration of the existence of anaerobic microsites and their relationship to denitrification activity is the work of Sexstone et al. (1985a, 1985b). Using oxygen microelectrodes, they measured oxygen profiles within saturated soil aggregates, detected anaerobic zones in their centers, and mapped oxygen contours.

The measured anaerobic radii were highly correlated with those calculated with the oxygen consumption-diffusion model, and measurable denitrification rates were associated only with aggregates that had anaerobic zones. However, denitrification did not occur in all aggregates that had anaerobic zones, probably because factors other than aeration limited denitrification.

bon 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 processes 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 focused on denitrification in soils of high pH.

Spatial Scale and Appropriate Controlling Factors. When considering the regulation of denitrification, it is useful to integrate the concept of spatial scale with the list of controlling factors (Fig. 14–8). Denitrifying bacteria are ultimately influenced by concentrations of oxygen, nitrate, and carbon compounds just external to their cell surfaces. Consequently, measuring these concentrations is appropriate for physiological studies in the laboratory. Other properties are likely to be more useful and insightful when studying denitrification in soils, particularly as the spatial scale increases from the microbial cell through microcolonies, soil aggregates, soil columns, and field plots up to the landscape scale (Box 14–7). We are just beginning to understand how to scale up such processes as denitrification.

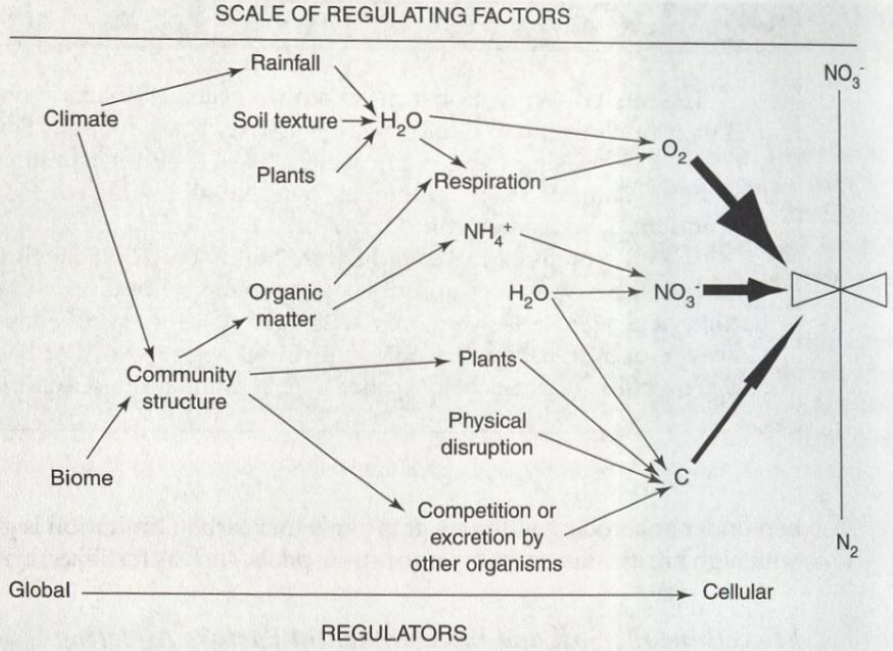


FIGURE 14–8
Regulation of denitrification at various spatial scales. The thickness of the right most arrows reflects the relative importance of oxygen, nitrate, and carbon as regulators of denitrification. *Adapted from Tiedje (1988). Used with permission.*

BOX 14–7

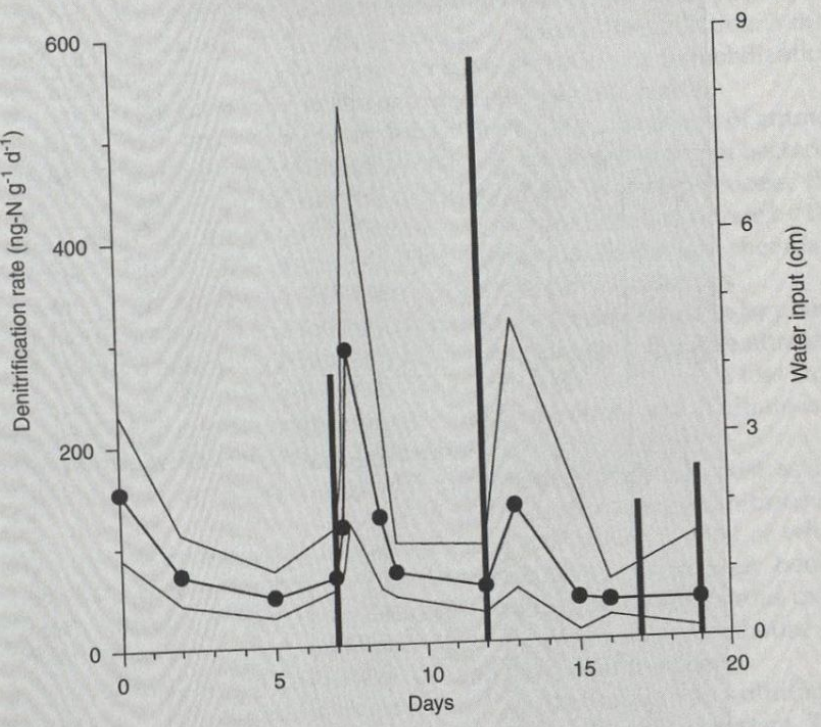
Spatial Variability of Denitrification

Denitrification is notorious for being highly variable in time and space. Denitrification rates can vary more than 100-fold from one day to the next.

Such short-term changes are often associated with precipitation, irrigation, or sometimes nutrient additions (e.g., inorganic fertilizer or manures). Seasonal responses, largely to soil temperature or precipitation patterns, are also observed. For example, denitrification rates in the Pacific Northwest of the United States are highest in the fall and spring when both soil temperature and water content are relatively high, whereas low rates are found in the winter, because of low soil temperatures, and in the summer, because of very dry soil conditions.

Variations in soil denitrification rates span several spatial scales, although the field plot level has probably been studied the most. The most common observation when sufficient numbers of denitrification measure-

BOX 14–7 *(continued)*

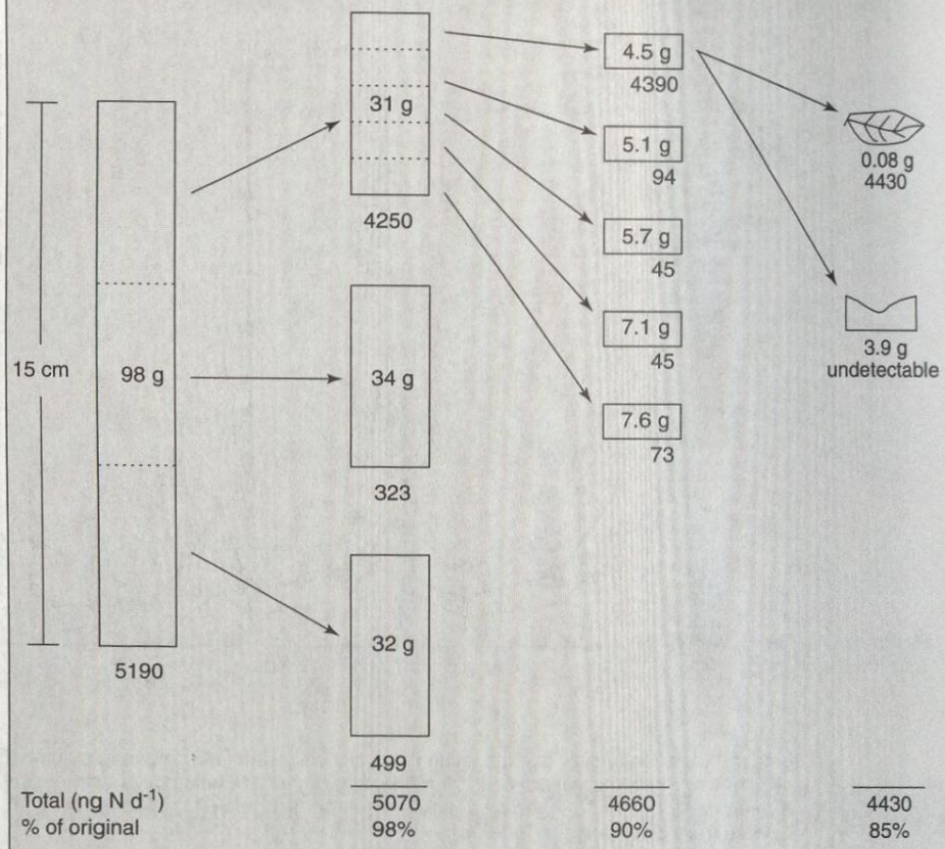


Temporal variation in daily denitrification rates of a clay loam in Michigan in autumn. The fine solid lines represent the upper and lower 95 percent confidence intervals and vertical bars represent water input. Note the response of denitrification to the addition of water. *Adapted from Sexstone et al. (1985a).*

ments are made is that most rates are low with just a few high or very high rates. This results in a skewed frequency distribution that is most often described as lognormal. This observation has often been attributed to the formation of "hot spots" of activity where optimal conditions of anaerobiosis, adequate nitrate, and available carbon concentrations coincide. The existence of such hot spots was perhaps best shown by the clever experiment of Parkin (1987). In this experiment, the denitrification activity of a soil core was monitored as it was subdivided into smaller and smaller units. Ultimately, over 85% of the activity of the core was found to be associated with one small piece of decaying vegetation.

(continued)

BOX 14–7 *(continued)*



Evidence for "hot spots" as important locations for denitrification in soil. The numbers at the bottom of each soil section are denitrification rates (ng-N day^{-1}). Note that almost all of the denitrification activity was associated with a single leaf.
Adapted from Parkin (1987).

SUMMARY

The nitrogen cycle is both fascinating and frustrating in its complexity. A thorough knowledge of the cycle is fundamental, though, if one wishes to understand the functioning of natural ecosystems, to manage agricultural ecosystems for productivity and sustainability, and to ameliorate environmental problems.

Most nitrogen in soil is in organic form. Organic nitrogen serves as a reservoir of nitrogen, slowly supplying the more dynamic and much smaller inorganic nitrogen pools. The conversion of organic nitrogen to inorganic nitrogen is

called mineralization. The first step of this process is the production of ammonium by ammonification, which is carried out by a wide variety of soil microorganisms and soil animals. Ammonification is always counterbalanced by the opposite pattern of immobilizing ammonium into the soil biomass through assimilation. A major controlling factor determining whether net mineralization or immobilization of nitrogen occurs is the C/N ratio of the decomposing organic matter.

The mineralization process is continued further by the conversion of ammonium to nitrate by the nitrifiers, which are a relatively restricted group of bacteria. Ammonia-oxidizing bacteria perform the first step of this two-step process, the transformation of ammonium to nitrite. Nitrite is further oxidized to nitrate by the nitrite-oxidizing bacteria. The ammonia and nitrite oxidizers are chemoautotrophic bacteria that gain their energy from these inorganic oxidations.

Nitrate has many fates in the soil environment. It is readily taken up by plants and can also be immobilized by heterotrophic microorganisms. Because nitrate is relatively mobile, it can be readily leached, which represents not only a loss from the system, but also a potential environmental problem. A final fate of nitrate is to be lost to the atmosphere through denitrification.

Denitrification occurs under anaerobic conditions, which can exist as microsites even in well-aerated soils. The reduction of nitrate to gaseous nitrous oxide and dinitrogen is accomplished by a wide range of bacteria, most of which normally function as aerobic heterotrophs. Denitrification is a relatively benign loss of nitrogen when dinitrogen is the dominant product; however, nitrous oxide production can be an environmental concern because it acts as a greenhouse gas and has been implicated in the destruction of ozone in the stratosphere.

The nitrogen cycle is closed by the process of N_2 fixation, which is ultimately the source of all the nitrogen that is transformed within the soil ecosystem. That process is covered in detail in Chapters 15 and 16.

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STUDY QUESTIONS

(Note, starred questions are more difficult.)

1. Draw your own diagram of the terrestrial nitrogen cycle including all major pools and transformations.
2. Describe the composition of soil organic nitrogen and how this relates to its biological availability.
3. Describe the steps involved in releasing ammonium from chitin.
4. A crop residue (45% C, 1.5% N) is chopped and mixed into soil. Assume that the soil microbial biomass has an average C/N ratio of 8 and an efficiency