THIRD EDITION

SOIL MICROBIOLOGY

ROBERT L. TATE III

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Soil Microbiology

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Third Edition

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to Ann, Robert, and Geoffrey.

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Preface

As I sit at my desk contemplating the appropriate words for this preface to the third edition of *Soil Microbiology*, a question arose of just why the rather long endeavor of writing another edition was necessary. Certainly, the basic tenets of the discipline are evolving, but not at a rapid rate. The objective of the first edition – "to provide the student with a strong basic knowledge of the biological, physical, and chemical properties of the soil and the microbes contained therein" – is unchanged. What has expanded significantly is our experience in using the concepts of soil microbiology to solve our expanding list of environmental problems. Thus, the most obvious differences between the previous editions and this one is the inclusion of information regarding soil microbial diversity and bioremedation of soil systems. Although the "central dogma" underlying these topics is still evolving, we can now include in a basic textbook the elucidation of the status of the topics and highlighting areas of deficiency in our knowledge base.

When I first began contemplating the task of writing this edition, I asked many of my close associates what they felt was the greatest need of the text (as I did for the second edition). Bioremediation and microbial diversity were frequently mentioned as possible topics but one response stands out in my mind. An individual whose opinion I highly respect strongly stated that no "new words" should be added. Students were already faced with a "mountain of information" to master. I must admit that I too agree, at least in part, with this observation. Students in my own classes commonly are overwhelmed by the vast amount of information before them. It is with this thought in mind that I went ahead and added more "words" to each of the chapters. My goal was to clarify the relationship of soil microbiology concepts to the general environmental matters with which students are experienced and to highlight new endeavors in the area of primary research. Since much of the foundation of soil microbiology derives from agriculturally based studies and our discipline remains a primary support for efficient production of food and fiber, the understanding of the agricultural environment is still a major part of our soil microbiology foundation. However, this foundation is now

greatly extended into more general soil concerns, such as urban soil management and soil stewardship and reclamation practices.

Lastly, I have tried to maintain the flavor of the first edition. Students need more than just a presentation of the "facts." Entries into the world of primary research literature are essential to provide a foundation for careers in our science. Thus, although complete review of the literature is not possible, an attempt was made to highlight a significant mass of current as well as historical research references. My apologies are offered to associates whose publications were overlooked, but such omissions are unavoidable under the page limitations for a basic text and to meet the objective of not overwhelming students with masses of new information. Students are challenged not only to explore the principles provided in *Soil Microbiology* but also to delve into the rich variety of primary research that has been amassed supporting the principles of soil microbiology and pointing the way to future endeavors.

I take this opportunity to thank my colleagues and students who have provided the inspiration and conducted the research that has made this book possible. In particular, I also thank the current members of my soil microbiology research group who have had to endure my deficiencies in providing true focus to their research endeavors as I put these words to paper. Their patience is gratefully acknowledged and highly appreciated. It is again my hope that this treatise will provide a basis for growth in the science of soil microbiology and an inspiration to the careers of young scientists.

Lastly, I must take the opportunity to thank Dr John Kelly. Our discussions and his contributions to Chapters 2 and 3 were indispensable. As with all of my interactions with John over the decades, he has provided essential insights into the nuances of environmental science.

Robert L. Tate III
New Brunswick, NJ
November 2019

Introduction

Soil microbes have been studied as interesting life forms. They also have been exploited as producers of substances of great societal importance, ranging from food and fiber to a variety of antibiotics. Agricultural-based research as well as basic research have provided a meaningful demand for monetary support. Today, environmental scientists, regulators, and the lay public are faced with the added demand for answers to microbiological concerns far beyond these basic science and agricultural needs. Examples of the extended informational voids include management of reclamation sites and the reparation of damaged soil systems, be they mismanaged or chemically contaminated.

As recently as the time of publication of the first edition of *Soil Microbiology*, justification for the primary interest in soil microbiology involved optimization of agricultural production or simply providing a vehicle for study of essential biochemical processes (e.g. DNA, RNA, and other basic biochemical concerns.). Indeed, not only is an appreciation of the biological processes occurring in soil essential to achieve societal goals of caring for terrestrial ecosystems but true wisdom in decision making is predicated on an understanding of how the individual soil biologically based processes combine to produce a vital, sustainable whole. The parts assembled into a viable soil system clearly produce a whole much more dynamic, much greater than can be predicted by their simple summation.

This thesis is underscored by the magnitude and multitude of anthropogenically impacted soil sites currently demanding some form of reclamation management; for example, refuse from energy production or mineral recovery form unsightly slag piles extending across the countryside. From within these piles, a yellow leachate with a pH of nearly 1 frequently flows. Nearly sterile soil and waters result from encounters with this deadly by-product of resource recovery. The questions become, "How best to prevent the production and leaching of the substances commonly yielded by acid mine drainage? How to restore the impacted soils and waters to functional, esthetic ecosystems? and How can the remains of

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the mining industry be managed to prevent further environmental degradation?" As will be revealed in the following text, these environmental concerns, although not fully resolved, have been replaced with more demanding concerns associated with climate change induced by basic soil microbially catalyzed by processes.

Another issue, perhaps less dramatic but of similar concern, is the problem of evaluating potential difficulties of amendment of soil systems with genetically engineered microorganisms (gems). The answers appear to be simple. An organic chemical has reached a soil system at toxic concentrations but no microorganisms capable of decomposing the toxicant exist therein. Yet, such microbes can be created in the laboratory through commonly available genetic manipulation procedures. Concerns involve unanticipated ecosystem degradation from the introduction of laboratory-created, alien organisms into established soil communities. "Will the introduced microbe survive sufficiently long to achieve the objective of its utilization? Will the unique gene carried by this gem be transferred to indigenous organisms, thereby creating an individual with capacity to wreak havoc on an otherwise stable soil system?" These questions represent concerns to which soil microbiologists must respond. Resolution of the conflict requires a clear understanding of the behavior of alien microbes within a functioning soil ecosystem as well as of the dynamics of gene transfer within soil populations.

These initial examples relate to environmental problems whose impact involves the reclamation or management of a limited region of soil. Solutions to environmental problems impacting the totality of our terrestrial system also rely on expansion of our knowledge and databases relating to soil microbial processes. For example, soil is a natural source and sink of greenhouse gases, such as methane, nitrogen oxides, and carbon dioxide. Basically, soil organic matter (humus) resources are the source and sink for these carbon compounds. Furthermore, the quantity of humus retained within a particular soil is the product of the physical, chemical, and biological properties of the system as well as any associated anthropogenic intervention. Therefore, all soil systems are characterized by occurrence of an equilibrium level of soil organic matter.

Anthropogenic intervention into the ecosystem can result in a shift in the quantity of carbon sequestered in the soil. This situation is nowhere more obvious than in soils developed for intensive agricultural production. Historically, the yield of carbon dioxide to the atmosphere due to reduction in the quantity of soil humus in these soils has been significant. Thus, a simplistic means of managing greenhouse gases that could be proposed is to alter the management of soil systems so that they become a sink rather than a source of atmospheric carbon dioxide. An appreciation of the potential benefits of this process can be derived from consideration of variation of soil humus levels resulting from the conversion of intensive cultivation practices into reduced till or no-till agricultural soil management. Unfortunately, the questions associated with assessing the role of soil in managing

greenhouse gases are more complicated. Decisions related to greenhouse gas management and associated terrestrial effects are impacted by, among other factors, the fact that soil temperatures are anticipated to increase due to global climate changes associated with the greenhouse phenomenon. Now an interaction of human soil management decisions, changes in the chemistry of plant inputs due to alteration of plant biomass composition by the elevated atmospheric temperatures, and alteration of soil physical properties (e.g. temperature and moisture) acting together create soil microbial community dynamics that are not as easily forecasted as was possible with alteration of agricultural soil management. An expanded comprehension of soil microbial dynamics and the effect of total-ecosystem processes on soil biological processes is needed.

It is with these concerns in mind that this treatise is presented. The overall goal is to provide the reader with a strong basic knowledge of the biological, physical, and chemical properties of soil and the microbial community therein necessary to provide the basis for sound environmental management and stewardship decisions.

1

Soil Ecosystems

Physical and Chemical Boundaries

Life in soil (e.g. active and resting of microbial species and its distribution within the soil matrix) is the product of direct and indirect interactions between highly variable physical and chemical matrices with associated equally variable biological communities composed of essentially all life forms. Soil biological communities are sustained across an intriguingly interesting span of chemical and physical conditions, especially when compared with the variability of the comparable properties of the environment supporting humans. Unfortunately for the scientists seeking a clear and accurate understanding of the identity and functions of soil microbes, the distribution of the microbes within the soil physical structure is not necessarily readily accessible. The location in soil most important for the function of soil microbes is not the macrosystem clearly accessible for scientific study but rather the microbial community which is impacted most by the properties of the microsites of a few cubic microns wherein they occur and function. Therefore, the primary goal of this introductory chapter of *Soil Microbiology* is to examine the general properties of soil and their impact on the associated microbial activity. To gain an appreciation of the complexity of the soil microbe's habitat, the overall physical and chemical properties of soil particulates controlling the development of soil communities will be introduced first.

Defining the world of the soil microbes: Microbes can clearly be studied in isolation from their native habitat, i.e. in soil samples of limited size or pure cultures of soil microbes incubated in the laboratory. Indeed, there is much still to be learned by such studies, but a full appreciation of microbes and their contributions to the sustainability of ecosystems can only be fully gained, or perhaps appreciated, by examining their capacity to function within their native habitat. Thus, we must initiate our examination of microbes in soil systems with a consideration of the basic properties of the soil environment itself. As will be demonstrated by the topics presented in this text, life is difficult for the microorganisms growing within the microsites comprising their soil habitat – thereby making "life" for the

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microbiologist attempting to understand the soil microbial community just as difficult, if not more.

Developing a clear understanding of the dynamics of soil microbial communities presents a formidable practical and conceptual challenge. The practical challenge is that due to the nature of soil itself, most of our endeavors to study the soil microbes must be conducted in the laboratory under less than natural conditions. The conceptual challenge results from the fact that many of the assumptions that we would make regarding the properties of that portion of the soil where the microbe grows are false. We see the general properties of a soil system such as a soil with a plentiful oxygen supply, a near neutral pH, and optimal moisture when in reality the microbes may be actually growing in a vastly different situation, perhaps in an acidic environment, in the absence of molecular oxygen, under water-limited conditions (e.g. arid or flooded soil). We, the observers, see the macro-world that we inhabit – the microbe functions within and is controlled by a few cubic micrometers of our world. Thus, the concept of the soil properties controlling microbial development and function can be, and most certainly are, vastly different from our picture of the soil system.

The soil ecosystem is the product of intricate interactions between a physical and chemical matrix of highly variable composition and biological communities composed of essentially all life forms. It is probably realistic to assume that a reasonable understanding of the extreme variability of microsites within the soil is a more modern development. Thus, a first task in studying soil microbial community development and function in soil systems is to expand our concept of the limits of physical and chemical conditions within which microbial life can exist. Thus, it is necessary to consider that the properties of the microbial world are variable within a range that extends far beyond that which is comfortable for us. Microbial life is sustainable across nearly the entire range of chemical and physical conditions existent on planet Earth.

A further limitation impacting the validity of our picture of how microbes function in soil is the fact that most of the data we have collected that is descriptive of their function are derived from laboratory-based studies examining individual microbial species or strains. Our ability to study the microbes directly in their "home" is limited at best. This simplification of soil physical, chemical, or biological properties, from several to perhaps a single function, has allowed for a reduction in the complexity of the system of study and of data interpretation. But at what cost – reduced understanding of the real functional complexity of the community? In part, to circumvent this limitation, more recently, use of consortia of bacteria and/or fungi for study of more complex processes, such as decomposition of xenobiotic compounds or changes in the diversity of the microbial community, has become the norm. These latter cultures exhibit some of the more complex interactions reflective of the soil

ecosystem. Use of defined cultures or even the simple mixtures of microbes making up consortia is generally justified by the conclusion that it is necessary to attain maximum control of experimental variables in order to elucidate clearly the processes of interest.

As an appreciation of the complexities of soils and their microbial populations is developed, the inadequacy of experiments using axenic cultures (that is, cultures composed of a single species or strain of soil microbes) or mixed cultures composed of a small number of microbial strains (that is, a consortia of soil microbes) to evaluate the world of the soil microbes becomes obvious. Generally, the basic properties of the biological processes measured in laboratory culture defy extrapolation to the more complex soil environment. Not only are stresses of the native soil system unmatched with defined culture conditions, but the microbes themselves change phenotypically and even genotypically in response to the laboratory growth conditions. Although the microbes studied may have been isolated from a sample of a soil, their metabolic capabilities and capacity to respond to their physical and chemical environment, as well as the presence of other microbes, may differ meaningfully from the strains existent in the original soil sample. That is, a microbial variant with appropriate properties for optimal growth in test tubes is usually selected either spontaneously (as a result of laboratory culture methods) or through genetic manipulation of the microbial isolates. The former selection results from the rich genetic variations within the genome of each individual bacterial species. It is easy conceptually to assume that each bacterial species present in soil consists of a group of individuals with essentially identical genomes. This viewpoint is far from reality. Bacterial species have similar traits and genetic composition, but their genomes contain many genetic variations (mutations).

Therefore, when members of a previously soil-resident bacterial species are selected for culture in the laboratory, mutant strains that previously existed in soil as a minuscule portion of the soil population of the species may be better able to grow under the laboratory conditions of the test tube than is the majority strain occurring in the soil community. Thus, a genetic variant becomes the dominant strain studied in the laboratory. Further selection of spontaneously occurring variants may – or more likely does – occur in the laboratory culture. As a result, data collected from experiments with isolated microbial species or strains frequently only explain or mimic the processes occurring in soil in part. Therefore, it is reasonable to conclude that soil microbiologists must expand the purview of experimental design to include the complexities – controllable and otherwise – of the total soil ecosystem. Therefore, the realm of soil microbiology must be defined to include both an understanding of the properties of the microbes themselves along with an appreciation of the impact of variability of the soil environment on these traits. Soil microbial ecology must involve an evaluation of the behavior of organisms in their native habitats. Even the

simplification of microbial community dynamics to those of a simple consortium is problematic, when we understand that the microbes growing in soil, rather than functioning within a colony or even a micro-colony, are more likely part of a biofilm that consists of several species or strains of bacteria with a variety of metabolic or physiological capabilities.

Are soil microbes really that important? To the purist, elucidation of the principles of soil microbiology is immensely interesting and fully justified strictly by the information generated relating to the basic ecological interactions occurring in soil systems. Just the fact that a single gram of soil can contain thousands of different microbial species is sufficient to pique our interest. Yet, due to societal management of terrestrial ecosystems, our concern for soil biological processes reaches far beyond just the realm of basic science. For example, from the world of environmental science, our myopic exploitation of natural ecosystems has resulted in situations where the functioning of resident microbial populations is precluded or severely limited by mismanagement. Spills of toxic organic chemicals result in scarred landscapes. Similarly, products of metal processing have resulted in sites resembling moonscapes (Figure 1.1a). Reclamation of such sites is attempted (e.g. Figure 1.1b), but for attainment of such objectives, management plans must be developed that allow for establishment of essential soil biological processes in soils containing extreme levels of toxic metals or a variety of organic toxicants. For the soils adjacent to a zinc smelter, attainment of improved microbial function required amendment of the soil with an organic matter source (sewage biosolids), liming the soil to raise the soil pH to above 7.0 (to immobilize the toxic metals), and planting the soil with a strain of grass that is capable of growing in soils with elevated metals loadings in order to stabilize the soil on the mountain slopes and reduce the soil erosion. Actually, the contributions of the grass to the microbial function extend far beyond just stabilizing the physical environment. By converting solar energy to plant biomass (photosynthesis), the plants provide the primary microbial energy source for the soil microbes.

In comparison with these obviously detrimental situations, development of soils for agricultural production may also reduce overall soil quality. Tillage initiates processes that may eventually reduce productivity of the soils. Loss of desirable soil structure and reduction of soil organic matter reserves are generally associated with implementation of intensive agricultural practices. Destruction of soil structure increases the bioavailability of native soil organic matter, a major microbial carbon and energy source. Conversion of the soil organic carbon to carbon dioxide reduces the soil productivity and sustainability, thereby reducing soil quality and increasing the production of a greenhouse gas.

A dramatic example of augmented soil organic matter decomposition due to cultivation of a previously undisturbed soil system is provided by observation of changes in soil properties resulting from cropping of peats. Development of these soils for agriculture initially required draining the water, resulting in conversion

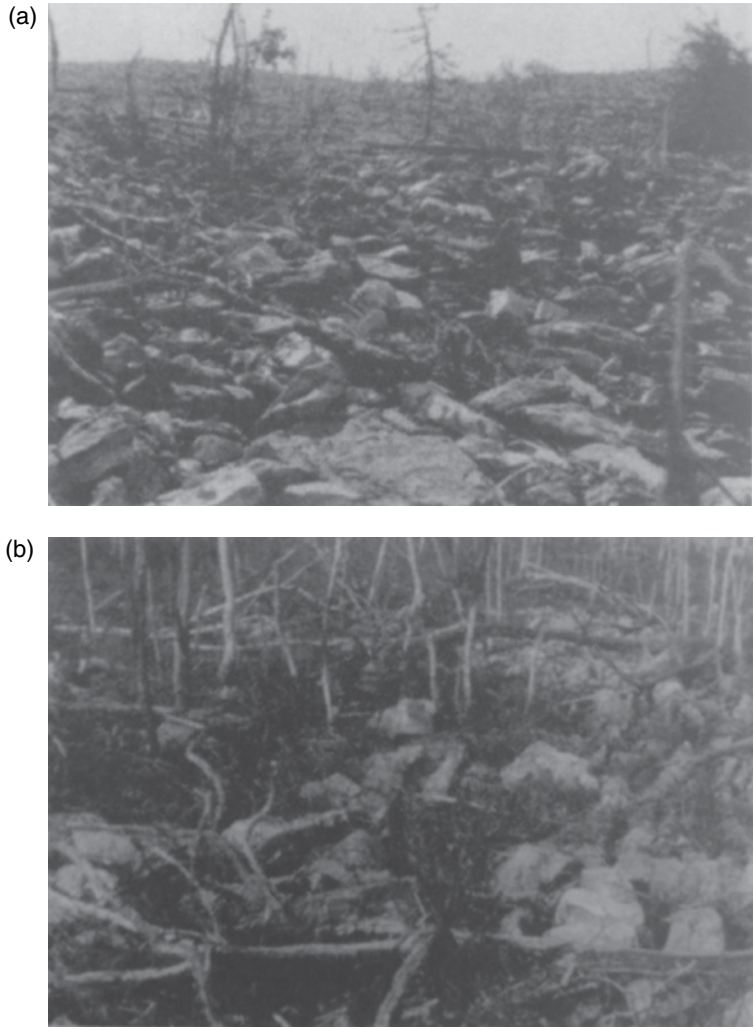

Figure 1.1 Metal-impacted site in Palmerton, PA (USA). (a) Site without reclamation management. (b) Site following amendment of soil surface with a mixture of sludge, fly ash, lime, and grass seed.

of the anoxic conditions to an aerobic state. This conversion of the generally anoxic swampy soil to one where molecular oxygen is no longer limiting resulted in stimulation of the aerobic heterotrophic bacteria. The microbes oxidized the plant debris that was accumulated under the swampy conditions prior to site drainage to carbon dioxide, mineral nutrients (e.g. mineral phosphorus and nitrogen), and water, resulting in loss of soil mass. The loss of the organic matter resulted in subsidence of the soil surface (Figure 1.2).

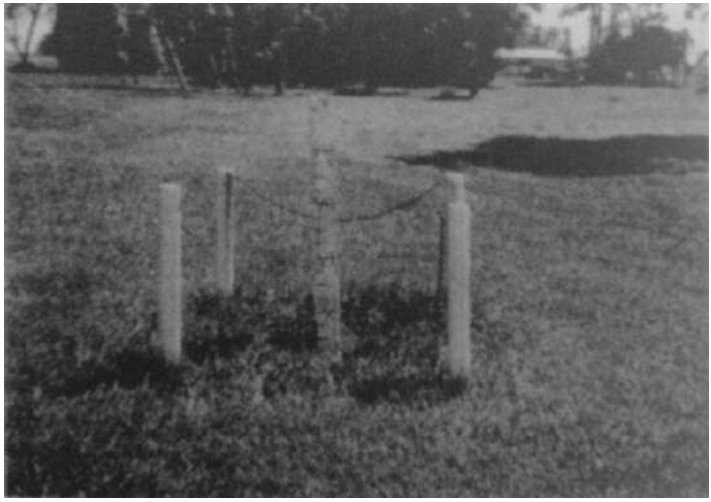

Figure 1.2 Subsidence pole located in the Everglades Agricultural Area of South Florida (USA) showing loss of soil elevation between 1924 and 1979.

In each of these examples – contamination of soil with metals, agricultural cultivation of the soil, or draining of swamps for agricultural production – the physical structure of the soil, its chemical properties, and the function of the biological community have been altered in a manner that reduces the quality of the soil system. Soil is a renewable resource, but the time frame for restoration of a badly damaged soil system may extend over several decades or even centuries. Thus, in the short run, soil must be managed with the philosophy that it is an exhaustible resource.

Proper soil stewardship cannot be achieved without a full appreciation of soil microbiological processes, for the totality of existence of a soil ecosystem relies upon sustaining a functional soil microbial community. To develop appropriate management plans for restoration of damaged systems or for maintenance of ecosystem productivity, a clear understanding of *in situ* biological processes is necessary. This cannot be fully accomplished by using data generated from the observation of bacterial populations growing at an unnatural rate under the optimized conditions of the laboratory culture flask. Realistic estimates of metabolic activity and the kinetics of the process in the microbe's native environment must be developed. Procedures necessary for maintenance of the soil chemical and physical factors controlling expression of microbial activity in a balanced state are needed to ensure the long-term survival of critical populations for ecosystem remediation and sustainability. Such factors as temperature, pH, moisture, surface area, osmotic strength, and presence of predators all interact to select for the development and survival of a stable microbial community necessary for long-term ecosystem function.

The challenge: Once it is understood that reality for soil microbes exists in their natural habitat, the primary challenge becomes defining that habitat. For the soil microbiologist, this is not an easy task. Microbes flourish in temperature-limited, native systems, under such extreme conditions as experienced in hot spring-impacted soils or in the cold desert soils of Antarctica. Highly productive soils may become biologically impoverished due to the impact of anthropogenically produced situations, such as those resulting from acid mine drainage or spills of xenobiotic organic compounds. Thus, it becomes clear that the soil ecosystem can be spoken of in general terms, but a true encounter with soil ecology must include an assessment of the length and breadth of these highly variable systems.

This assessment must start with differentiation of the entity to be studied. Soil microbiologists must realize that they are studying specific soil types, not simply "soil." The commonly heard statement among soil microbiologists, "Soil was used in our experiments," would sound as strange to a soil scientist as would "A bacterium was studied" would to a biologist. Currently, the US soil classification system divides world soils into 12 orders (based on soil properties that reflect soil development). Examples of soil orders are aridisols (dry soils), andisols (volcanic soils), gelisols (permafrost-impacted soils), histosols (organic soils), inceptisols (embrionic soils), spodosols (typical of forest systems), and vertisols (characterized by soils with high-swelling clay contents). These orders are subdivided into suborders, great groups, subgroups, families, and series. Currently, over 15 000 soil series have been identified. (See Brady and Weil 2017 for further discussion of soil taxonomy.) This situation of defining the specific soil unit of study is further complicated by the fact that a given field site may contain one or more soil series. Furthermore, biological activity within the same soil series varies with such ecosystem depending on properties as plant type and distribution, seasonal climatic variation, and management practices – current and historic. Yet, the properties of the soil type underlying an ecosystem as defined and impacted by the aboveground communities must be assessed and understood.

1.1 Soil as an Ecosystem

The properties of a soil system are not simply a summation of the general properties of its individual physical, biological, and chemical components. They are the result of the individual properties as modified by their interaction with each other as well as their distribution within the soil matrix. These interactions result in a soil ecosystem that is truly much greater than just the sum of its parts. For example, association of the soil mineral and organic materials in soil aggregates results in co-occurrence of anoxic and aerobic biological processes within a few millimeters or less of each other in the surface horizon of a soil permeated by our oxygen atmosphere. As will be elucidated in Chapter 2, even limited growth of a pioneer

population of microbial cells can result in modification of the microbe's environment sufficiently to enhance future growth and community development. Thus, a soil ecosystem could be said to be evolving toward an optimized ecological state dictated by the interactions of each of the soil components.

Therefore, any analysis of the structure of soil systems and the principles describing their function must commence with an evaluation of (i) the soil physical components, (ii) their assembly into the physical whole, and (iii) the implications of this structure on soil biological processes. The study must then proceed to a determination of the feedback mechanisms involved with living populations interacting with their physical environment. This analysis is necessary to develop fully the principles or maxims describing microbial interactions in soil and their importance to that portion of the system most commonly regarded esthetically – the aboveground plant and animal community.

1.1.1 Soil System Function

A soil's classification could be based on an understanding of the applicable physical, chemical, and biological properties of soil that make each soil unique. For example, soil definition could be based on our understanding of the impacts of plant community development on soil ecosystem properties whereas others studying the soil system could emphasize the variation in general soil properties, emphasizing an appreciation of the foundational role of soils in societal development, function, and sustainability. Indeed, societies can rise and fall based on the capability of their soil to provide ecosystem services (French 2010). Since soil microbes are key participants in the provision of these services, societies are truly built upon the function, sustainability, and diversity of the soil microbial community. To fully appreciate the intricacy of this soil microbial community, our more general image of the material that we walk on, build our infrastructure on and depend upon for food and fiber production must be enlarged to include a picture of the microscopic world in which soil microorganisms live and function.

The soil physical structure and soil microbes: Most soils are classified as mineral soils – that is, soils that are composed primarily of mineral matter and whose physical properties are controlled by the proportions of the mineral particulates contained therein. These soils generally contain from less than 1% to approximately 4% organic matter, but they may consist of as much as 20% soil organic matter. Additionally, an organic layer up to 30 cm in depth may be found on the soil surface (e.g. a forest litter layer). These soils are to be contrasted with the much less commonly encountered organic soils (histosols). Organic soils contain more than 20% organic matter as exemplified by peats and mucks.

The most obvious components of mineral soils are sand, silt, and clay, each of which is defined by its physical dimensions (Table 1.1). Note that this separation

Table 1.1 Size distribution of soil particle classes

Particle class	Subclass	Mean diameter (mm)
Sand	Very coarse sand	2.00–1.0
	Coarse sand	1.0–0.5
	Medium sand	0.5–0.25
	Fine sand	0.25–0.1
	Very fine sand	0.1–0.05
Silt		0.05–0.002
Clay		<0.002

Source: USDA Public Domain.

of soil particles is based on particle size only. For example, the clay fraction is strictly defined as having a mean diameter of less than 2 μm. Therefore, this fraction will contain clay minerals plus other soil components, which are not necessarily clay minerals but are of comparable size. A soil is classified into a specific textural category by the quantities of the mineral separates that it contains (Figure 1.3)

This disposition of soil by textural classification is useful for characterizing a particular soil of interest (either for field or laboratory experiments), but it is in reality the soil structure – that is, how these individual components are assembled – that best defines the habitats of soil microorganisms. Two soils may have nearly identical textural compositions yet exhibit extremely contrasting microbial activities. For example, microbial properties of a silt loam in an area flooded with acid mine drainage waters would reflect the prevailing stresses of oxygen deprivation and extremes of acidity, whereas a silt loam from a well-maintained agricultural site would be free of such limitations. Furthermore, a soil with a well-developed aggregate structure in contrast to one where the structure has been lost due to intensive cultivation would be more conducive to microbial community development.

Clearly, a soil classification system is required that is descriptive of *in situ* ecological influences on biological properties. This information is provided by soil classification systems, such as those of the United States Department of Agriculture (USDA) or the Food and Agriculture Organization of the United Nations (FAO). Within these taxonomic systems, soils are grouped by field and laboratory properties, among which are soil texture, moisture, pH, temperature regimes, and horizon development.

Soil profile development: An appreciation of soil horizon development and its effect on soil microbial activity is necessary to develop a more complete picture of the impact of soil biological processes on the properties of an ecosystem. A soil

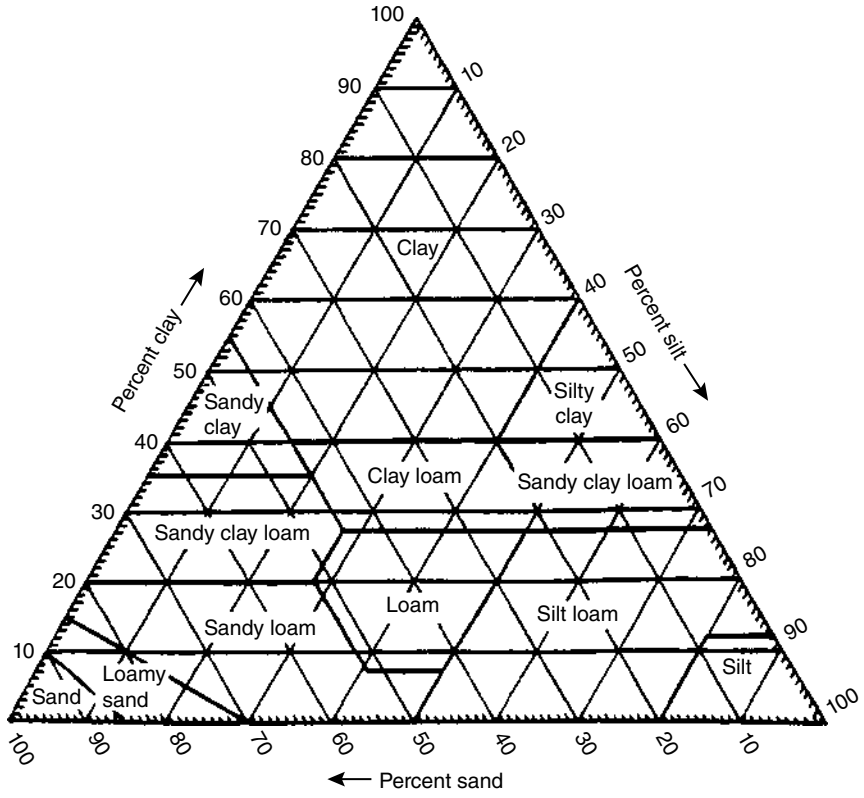

Figure 1.3 Designation of soil textural classes by principal mineral component analysis. *Source:* USDA Public Domain.

horizon is a layer of soil approximately parallel to the land surface and differing from adjacent layers physically, chemically, and biologically, or in characteristics such as color, structure, texture, consistency, biotic populations, and pH. Soil horizonal development is clearly a product of current ecosystem properties as well as of site history. Each soil subgroup description includes an analysis of horizonal structure. Microbiological properties of a specific horizon may be studied in isolation from other portions of the soil profile. In such studies, the designated portion of the soil profile may be defined as an ecosystem in itself.

The most commonly studied of soil horizons in microbiological research are the O, A, and B horizons, although as a result of complications of groundwater pollution, considerable research effort is being expended to understand biological processes occurring in deeper-lying aquifer materials. O horizons are dominated by organic material and are exemplified by forest litter layers. The A horizons are mineral layers formed

on the soil surface or below the O horizon. These regions are characterized by accumulations of organic materials intimately associated with soil mineral matter. In many situations, these organic materials are a product of plant community development in native, unmanaged ecosystems. A horizons are usually spoken of as surface soils. Colloidal organic matter concentrations tend to be maximized in this portion of the mineral soil profile. B horizons are usually formed below an O or A horizon and are dominated by (i) carbonates, gypsum, or silica, alone or in combination; (ii) evidence of removal of carbonates; (iii) concentrations of sesquioxides; (iv) alterations that form silicate clay; (v) formation of granular, blocky, or prismatic structure; or (vi) combinations of these. In soils without organic surface accumulations, microbial activity tends to be maximized in the A horizon and declines precipitously in the B horizon.

1.1.2 Soil Formation and the Microbial Community

Soil microbiologists frequently report results of study of a limited number ofsoil types and provide minimal descriptions of the sites from which the soil samples were collected. It is not unusual to see statements such as "a garden soil was used" in the literature. From the foregoing discussion, it is apparent that an understanding of not only the currently existing physical and chemical properties of the soil but human-imposed as well as natural events influencing soil formation must be examined. Furthermore, primary goals of research include not only improvement of our understanding of the function of the local soil site but also elucidation of the soil science principles that will increase our understanding of soils in general. Indicating the ecosystem type from which a particular soil sample was collected is generally inadequate to allow proper interpretation of the data, extrapolation of the principles revealed by the research, and, frequently, replication of the study by other researchers asking similar scientific questions. Although it may seem adequate to report that a grassland soil was evaluated in a particular experiment, application of proper soil classification procedures may reveal that a variety of soil types occur within the region classified by an ecologist as a grassland. That is, to conduct a meaningful examination of a soil ecosystem, the area to be sampled must be appropriately described. An appreciation of the variation of soil properties and the importance of the variation to the ecosystem services provided by the soil microbes can be gained by a short consideration of the controllers of soil genesis.

State Factor Theory of soil development: The effects of interactions of total ecosystem properties on soil development were described succinctly by Jenny (1941) in his State Factor Theory, frequently referred to as the CLORPT model. The original model stated that soil development is a function of climate, organisms, relief (topography), parent material, and time. The theory was subsequently modified to include human influences (Amundson and Jenny 1991). Although the model

emphasizes the major factors affecting soil formation, possibly primarily from a macro-environment view, it is reasonably clear that each of the factors affects and is affected by the soil microbial community. The synergistic associations of soil microbes and their physical and chemical environment determine the properties of all soils involved as well as the ecosystem services derived from the soil. The effect of some of the factors, such as climate, on soil microbes is clear, especially when comparing such extremes as desert and forest ecosystems or even boreal and tropical rainforests, whereas others such as relief are less obvious. Perhaps the latter effects on microbial function could be clarified by consideration of variation of temperature and moisture on east versus west aspects of a hill, where soil temperature would vary due to different impacts of morning and afternoon sun exposure or direction of prevailing rain storms.

In regard to human impacts on soil formation, at one point in human history agriculture could have been considered to be the primary controller of soil development (e.g. destruction of soil structure, alteration of nature and properties of organic matter inputs) but now it is reasonably easy to accept the observation that the genesis of all soils is directly affected by human society (e.g. distribution of pollutants, variation in air and water contents, as well as alteration of climate via anthropogenic-imposed climate change or even drainage of large swamps). In all cases, the dynamics and nature of the soil microbial community are determined at least in part by the evolving soil properties as much as the evolution of the soil properties are determined by the function of the soil microbial community.

Interrelationship of microbial communities throughout the soil profile: A complication to description of soil systems results from the fact that soils are continually changing. To the casual observer, it may seem that soil types and their properties represent a constant in their conceptualization of an ecosystem. In reality, as can be concluded from the CLORPT model, soil physical, chemical, and biological properties are continually changing. Soil pH, salinity, aggregate structure, and cation exchange capacity are but a few examples of soil properties constantly evolving due to the actions of the biological community (plant, animal, and microbes). For example, microbes produce organic acids, carbon dioxide, and hydrogen ions that alter the soil pH, thereby affecting the solubilization of soil minerals and ultimately the salinity of soil water. Additionally, as a result of variation in the composition and production of root exudates, aboveground differences in plant community type and density can cause variations in belowground microbial and enzymological activities that may range over several orders of magnitude. This effect of plant biomass productivity on soil enzymes is exemplified through analysis of enzyme activity in a reasonably uniform organic soil (i.e. a single soil type) in the Everglades Agricultural Area (South Florida, USA). Acid phosphatase, invertase, xylanase, cellulase, and amylase activities varied by as much as 50-fold between not-cultivated and soils cultivated to sugarcane,

St Augustinegrass (*Stenatophrum secundatum* [Walt] Ktze.), or paragrass (*Brachiaria mutica* [Forsk.] Stapf) (Duxbury and Tate 1981). A limited understanding of the complexities of these organic soil-based systems would result if each soil sample (uncultivated or from grass or sugarcane fields) were simply classified as agricultural soil. Inputs from the aboveground plant community necessitate dividing the study area into at least three ecosystem types. Note also that in the study cited, all aboveground biomass could have been grouped into a category of grasslands (i.e. both sugarcane and the St Augustinegrass are by definition grasses).

Although plant communities are instrumental in soil development, it must be stressed that soil properties can be very different in seemingly related ecosystem types. This situation is exemplified by comparing two large forest groupings: temperate and tropical forest soils. Temperate forest soils may be moderately acidic (pH 3.5–4.5) and possess a well-defined horizonal development (Figure 1.4). They may also have a surface litter layer plus a subsurface horizon (spodic horizon) with an accumulation of organic matter. Clearing of forests and cultivation of such soils in the temperature regions has resulted in moderately productive agricultural systems. In contrast, tropical rainforest soils generally lack a surface

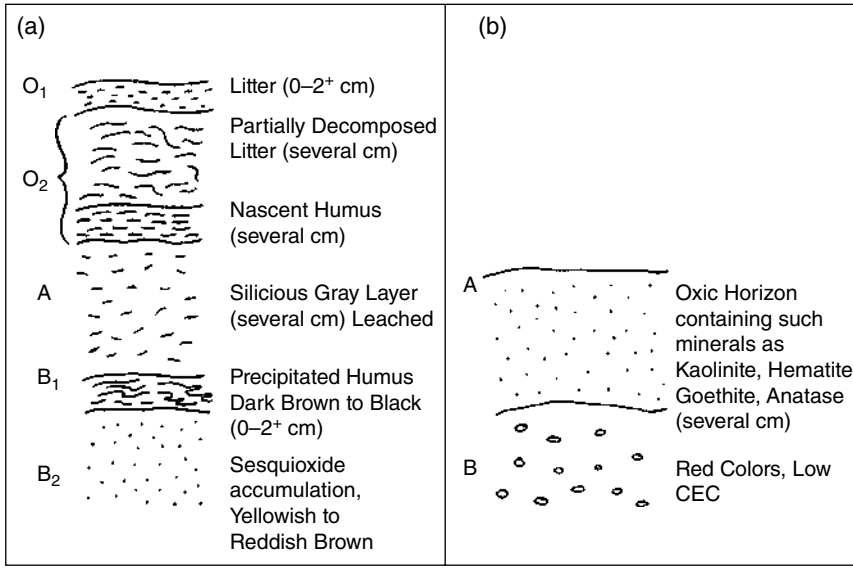

Figure 1.4 Comparison of idealized soil profile models for temperate and tropical forest soils. (a) Spodosol surface horizons with accumulated organic matter, and leached A horizon. Spodosols are exemplified by acidic forest soils. (b) Latosilization as would be seen in an oxidol (tropical forest ecosystem). These soils are generally highly leached, acidic soils with no significant humus layer. See Fanning and Fanning (1989) for further discussion of these soil types.

organic horizon; are usually highly acidic, nutrient-poor soils; and can be poor candidates for agricultural development.

Thus, it may be concluded that our operational definition of a soils must include the concept that they are composed of unconsolidated mineral or organic matter, as stated above, but it must also be understood that general ecosystem properties affect soil property evolution. The microbiologist must realize that soil is a continuously developing entity with properties reflective of the site in which it has evolved and is still evolving. A corollary to this maxim is that significant changes in the overt soil properties will meaningfully affect the nature and properties of the soil microbial community. Ultimately, when characterizing a soil ecosystem, the history of the soil must be considered along with the current situation.

1.1.3 Implications of Definition of the Soil Ecosystem

Generally, understanding the effect of microbial/soil mineral interactions is important for developing local soil management plans, but a more general goal of studying soil processes could be to elucidate the underlying principles of soil development, enabling extrapolation of the data to larger soil systems or even soils in general. The ability to extrapolate experimental findings to a larger venue depends on the extent of the soil system selected for study. Frequently, the greater the variability of physical or chemical properties in the soils and the better the principles underlying their impact on biological properties are elucidated, the greater the applicability of the discoveries to our understanding of the properties of soils in general. Unfortunately, simply because of the logistics involved in soil sampling and laboratory analyses, arbitrary limitations of the extent of the ecosystem area studied are inevitable.

Variability of soil properties within even a more limited field site is such that a basic requirement of research projects is that the soil samples must reflect site variation. The better our understanding of soil variability, the better the potential for elucidating the full range of the microbial processes occurring therein and the properties of the soil controlling these activities. Conversely, the smaller the portion of the overall system studied, the less the potential for valid extrapolation of the data to provide a description of the total ecosystem. Thus, a soil microbiologist faces the dilemma of selecting a soil site small enough to be sampled in a representative manner without being overwhelmed by the number of soil samples to be studied, but sufficiently expansive that the data collected will be at least to some degree representative of the total ecosystem. The more limited the dimensions used to define an ecosystem of consideration, the more important becomes consideration of the impact of external processes on the integrity of the system. For example, a B horizon soil may be examined, but it must be considered that energy derived from oxidation of organic soil components depends to a large degree on

the rate that the fixed carbon is leached from the A horizon to the B horizon. Drawing boundaries that define the portion of the larger ecosystem to be studied will clearly enable some aspects of the ecosystem to be self-contained (that is, be treated as a closed system) whereas others must be supplied from external sources (that is, function as an open system). A good example of this situation involves the aerobic microbial populations deriving their carbon and energy from oxidation of organic carbon (the heterotrophs). In the vast majority of situations, the carbon oxidized for energy originates from beyond the world of the bacterial microcolony. It is produced photosynthetically by aboveground plant communities.

1.2 The Micro-ecosystem

Existence of aboveground communities literally rests on the stability and function of soil processes occurring at the microscopic level. Because microorganisms range in size from less than a micron to a few microns, individual soil particles have a major impact on their function. The challenging aspect of this observation is that the biological processes occurring at the micron level can have profound implications on total ecosystem development and longevity. For example, a series of bacterial colonies consisting of as few as a half-dozen cells each may determine the fate of a potentially ecosystem-destructive xenobiotic compound or the bioavailability of toxic metals, such as mercury or arsenic. Thus, a clear understanding of the interactions of the soil microbial community and elemental soil components (sand, silt, clay, and colloidal organic matter) is imperative to comprehend total ecosystem function.

1.2.1 Interaction of Individual Soil Components with the Biotic System

Surface area and charge density have major effects on microbial and enzyme activity in soil. The basic components of soil are sand, silt, clay, and humic substances. Although all soil particulates provide surfaces for microbial colonial development, clay and colloidal organic matter have the smallest diameters and therefore present the largest surface area for interaction with soil microbes and their products. The relationship between estimated particle diameter and surface area is demonstrated in Figure 1.5. Due to their colloid size and the fact that humic acids and most clays are general negatively charged, these two soil physical components are major determinants of the extent of activity of soil microbes, enzyme activity and availability of their substrates. Thus, the nature and properties of these soil components must be examined in greater detail.

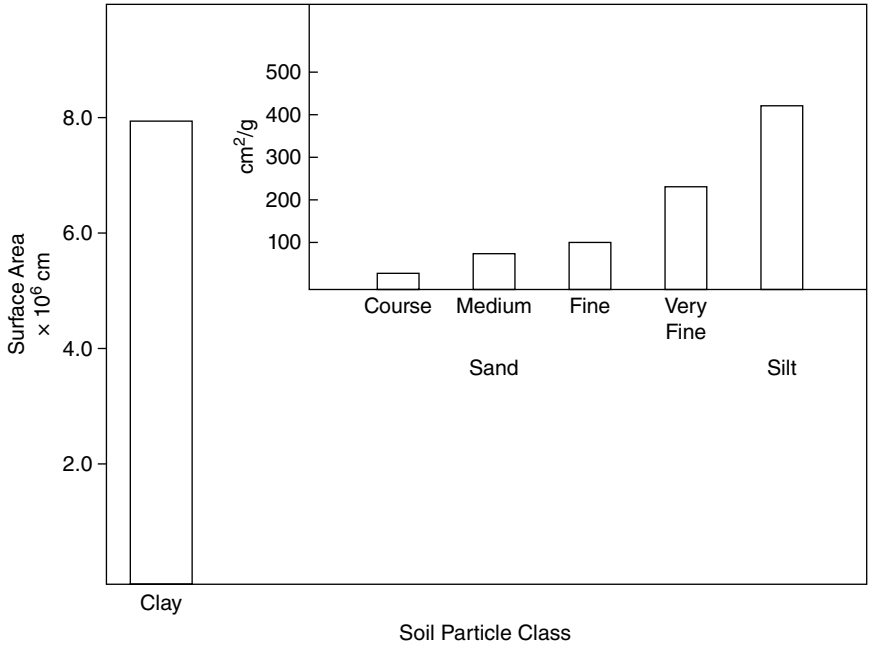

Figure 1.5 Variation of surface area of soil particles: The particles are assumed to have spherical shapes. *Source:* Data are selected from Foth and Turk (1972).

1.2.1.1 Clay and Ecosystem Function

The high negative charge and high surface area g^{-1} clay result in the clay particles having a unique impact on the soil biological and chemical processes. The negative charge of 1 g of clay particles is at least 1000-fold that of 1 g of sand (Figure 1.5). Thus, even if the clay particles constitute as little as one-tenth of the mass of soil particulate matter, they have a controlling role in soil biological, chemical, and physical processes. Soil water relationships provide an excellent example of the nature of these clay-driven interactions. Soil microbes are commonly associated with the surface of soil particles. Water films are distributed around the soil particles. Due to the physical interactions between the bipolar water molecules and the soil particle surfaces, water molecules closest to the clay particle surface are held more tightly to the clay particles than they are to each other in the water layer, so are less available to the microbes (see Brady and Weil (2017) for further description of the role of hydrogen bonds and dipole moment in these processes.) This negative charge is sufficient to create water – clay layering in the area adjacent to the clay particles. Water – water layering occurs further out from the particle surface to result in a clay – water + clay − water layering effect. In soils with more surface areas and high negative charges, more water is needed to coat the particles

before a sufficiently thick water layer on the clay particles for the microbes and growing plants is formed. Again, refer to Brady and Weil (2017) for further information regarding these processes and interactions.

Considering the disproportionate impact on soil processes that results from the colloidal size/high surface area of clay minerals, it can be concluded that an examination of basic clay properties is key to understanding microbial function in soils. Clay minerals are secondary minerals; that is, except for mica, clay minerals are formed in soil from the weathering of primary minerals or from the products of their weathering. Most clays are crystalline and are composed of layers of oxygen atoms associated with silicon and aluminum atoms through ionic bonding. For crystalline clays, these silicate and aluminum layers are stacked (like a deck of cards) into clay particles, termed *micelles*. As will be noted below, less regularly oriented amorphous clays are also found in some soils. Common groups of clays are layer silicate clays, hydrous oxides of iron and aluminum, plus allophane and other amorphous clays.

Layer silicate clays are primarily crystalline clays with specific structures and predictable properties. These clays have a plate-like structure formed from flat sheets of silicon, aluminum, magnesium, and iron linked by oxygen and hydroxyl groups. The major silicate clays are commonly referred to as phylosilicates due to their leaf-like structure. The crystalline structure can be summarized as being composed of two distinct crystalline sheets.

- A silica-dominated sheet composed of silicon atoms surrounded by four oxygen atoms in a tetrahedryl arrangement. The interlocking array of silicon and shared oxygen atoms is a tetrahedron sheet.
- An aluminum- or magnesium-dominated sheet composed of aluminum and magnesium atoms surrounded by six oxygen atoms in an octahedryl arrangement. Thus, the layer is referred to as an octahedryl sheet. If the layer is dominated by aluminum, it is a dioctahedryl sheet, and if by magnesium, a trioctahedryl sheet.

The tetrahedryl and octahedryl sheets are bound together in regular crystals by shared oxygen in the different layers. Major groups of these layer silicate clays are:

- 1 : 1 – one tetrahedryl (Si) sheet per octahedryl (Al) sheet. Kaolinite is the most prominent member of this group
- 2 : 1 – one octahedryl sheet sandwiched between two tetrahedryl sheets as exemplified by smectite (montmorillonite)
- 2 : 1 : 1 – typically 2 : 1 crystals as above alternating with Mg-dominated trioctahedryl layers as exemplified by chlorites

Both a variable (depending on soil pH) and a fixed negative charge are associated with layer silicate clays. The pH-dependent charge results from ionization of the hydroxyl groups on the surface and edges of the crystal. The fixed charge is the product of isomorphous substitution for the aluminum or silicon atoms within

the crystalline structure. For example, the aluminum atom is only slightly larger than the silicon atom. Therefore, aluminum can fit into the center of the tetrahedron in place of the silicon without altering the basic physical structure of the crystal. The aluminum atom carries a +3 charge compared to the +4 charge of the silicon. Thus, one of the negative charges of the shared oxygen atoms is not satisfied, resulting in a net negative charge of the crystal.

The negative charges of clay minerals contribute to the soil cation exchange capacity and are instrumental in interactions with soil organic components and microbes. The quantity of negative charges associated with clay minerals is a property of the specific clay type. Thus, identification of the type of clay in a soil site is essential for predicting the chemical and biological dynamics occurring therein.

Gibbsite ($Al_20_3.3H_20$) and goethite ($Fe_20_3.H_20$) are examples of hydrous oxides of iron and aluminum. These clays are found primarily in the highly weathered soils of the topics and subtropics, but also occur in temperate regions. They are less studied than the layer silicate clays. Hydrous oxides of iron and aluminum may be crystalline or amorphous. They are not sticky clays and their charge varies with pH. At high pH, they have a slightly negative charge, whereas at the highly acidic pH values common to the weathered tropical and subtropical soils where they generally occur, they are positively charged. This contrasts to the layer silicate clays, which are normally negatively charged.

Allophane and other amorphous material clays are much less studied than the two other clay mineral groups. The most significant members of this class of clays are allophane and imogolite. They are aluminum silicates and are prevalent in andisols (volcanic ash-derived soils). Thus, examples of locations where they could be encountered are the Northwestern United States, Hawaii, and Japan.

Adsorption of organic substances to clay particles: Clay minerals provide a surface for sorption of a variety of organic substances. Not only do whole microbial cells and viruses sorb to soil clay particles, but also a diversity of organic compounds have been demonstrated to interact with clay surfaces. Examples of the types of organic compounds reacting ionically with clay particles include carbohydrate phosphates (Goring and Bartholomew 1951), nucleotides (Goring and Bartholomew 1952), humic and fulvic acids (Greenland 1971; Kodema and Schnitzer 1974) and aromatic compounds as exemplified by p-cresol (Boyd and King 1984). Most biologically produced compounds interact with clay particles. The environmental quality concerns relating to clay–organic matter interactions involve the bioavailability of the sorbed substances and, for biodecomposable substances, the impact of sorption on degradation kinetics. Tightly sorbed organic substances, especially those existing within soil micropores, may be essentially unavailable to the biological community, i.e. they may be sequestered. Biodegradation susceptibility of sorbed organic compounds may be reduced or enhanced. Thus, the adsorption potential of any organic compound entering soil should be evaluated in relation to estimation of the effect of this process on its longevity *in situ*.

It must be emphasized that both substances entering the soil system from external sources (e.g. pesticides and other soil amendments) and those synthesized *in situ* may be sorbed onto clay surfaces. Sorensen (1972) found that amino acid metabolites synthesized in montmorillonite-amended soil following cellulose, hemicellulose, or glucose amendment were bound by soil clays. Clay-amended soil retained two–threefold more carbohydrate-derived amino acid carbon than did nonamended soil over a three-year incubation period. In a related study, ^{14}C-labeled cellulose was added to seven soils with silt plus clay contents ranging from 8% to 75% (Sorensen 1975). As in the previously cited study, the quantities of amino acid carbon synthesized from the cellulose remaining in the soil after 30 days of incubation ranged from 6% in the lowest silt plus clay-containing soil to 18% in the highest silt plus clay-containing soil, implying a stabilizing or protective effect due to sorption to clay minerals. This stabilizing effect by clay minerals on organic matter is of particular interest when considering the potential for mitigating the climate change potential resulting from mineralization of soil organic matter.

Association of organic compounds with clay particles is not a totally random process. There is an enrichment of specific carbon moieties within clay particles. This is exemplified by evaluation of the properties of humic acids associated with clay particles. In native soil samples, aliphatic structures are more common in humic acids derived from the clay fraction than other soil fractions (Arshad and Lowe 1966; Schnitzer et al. 1988; Schulten and Schnitzer 1990). Using chemical, infrared, and ^{13}C nuclear magnetic resonance (NMR), and gas chromatographic mass spectrometric analysis, Schnitzer et al. (1988) found that about half of the clay-associated organic matter consisted of humic materials, the remainder being mainly long-chain aliphatics.

Clay–organic matter adsorption mechanisms: An immediate conceptual problem raised by this list of materials sorbed to clay particles is the observation that both the most commonly occurring clays in temperate region soils and many of the sorbed organic compounds are negatively charged at the predominant soil pH. Thus, mechanisms must exist to reduce the repulsive forces between the like charged entities. Examples of such mechanisms are reduction of the quantity of negative charges (pH modification or methylation of the carboxyl groups on the organic matter) and cationic bridging of negatively charged functional groups of the humic substances with the clays. The latter process is depicted by a model proposed by Greenland (1971) (Figure 1.6). The negative charges on the organic and mineral materials are linked in soils by divalent cations.

Organic compounds retained on clay particles through cationic bridges can clearly desorb from the surface with changes in ionic strength and cation content of the soil solution. A more permanent association of organic compounds to clay particles can occur. This association between clay particles and organic materials results from interlayer clay–organic matter complex formation. For example, this

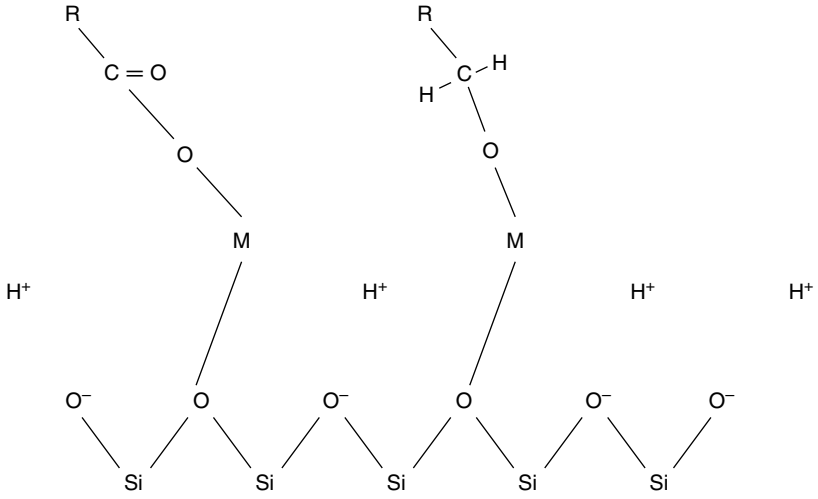

Figure 1.6 Schematic drawing of divalent metal (M) salt bridge linking of the surface of a mica type clay with a negatively charged organic compound and the distribution of a hydrogen ion cloud around the clay surface.

was demonstrated to occur in two New Zealand soils by Theng et al. (1986). Humic substances with a polymethylene chain structure were found to be regularly interstratified with mica-smectite in highly acidic soils containing low microbial activity.

Any process that alters the negative charges of either the clay or organic matter reduces the repulsive forces. This can be accomplished by altering soil pH or through methylation of the carboxyl groups. For example, Nayak et al. (1990) demonstrated that reduction of the acidic groups of humic substances through methylation increased fixation with montmorillonite. The increased association with the clay particles resulted not only from a reduction of interparticle repulsive forces but also from a reduction in hydration energies.

Biodegradation and clay interactions: It has long been understood that the association of clay and soil organic matter affects the decomposition rate of sorbed organic compounds. For example, early studies of the impact of clay on decomposition of a wide variety of organic compounds have shown that purified clay minerals when added to growing microbial cultures can stimulate, decrease, or have no effect on decomposition of organic materials (see Hwang and Tate 1997; Kunc and Stotzky 1974). Kunc and Stotzky (1974) found no clear relationship between the type of organic substance and the type of clay relating to the effect on biodegradation except with aldehydes, whose decomposition was accelerated by clay minerals. Lynch and Cotnoir (1956) noted that bentonite inhibited decomposition of a variety of substrates but that illite and kaolinite had little effect on decomposition.

Similarly, Olnes and Clapp (1972) found that the kinetics of the decomposition of the montmorillonite-dextran complex contained periods that were affected to a greater degree by the montmorillonite content, whereas other portions of the degradation curve were affected more by the dextran concentration.

Clearly, it is difficult to make general statements regarding the impact of clay sorption on biological decomposition kinetics. The basis for these seemingly contradictory observations can be revealed by examining the effect of clay on bioavailability of sorbed organic compounds. Two ramifications of clay–organic matter interaction can be pictured depending upon the relationship of the quantity of substrate present in the system and the ability of the organism to interact with it. Carbon and energy sources for microbes may exist in their ecosystem at concentrations too low for significant interaction between them and the microbes to occur. These materials may become sufficiently concentrated on the clay surface that the quantities desorbed from the clay and thus occurring in the water surrounding the clay particle are sufficient to induce microbial catabolism. In contrast, for substrates present in high concentration in soils with high clay contents, their concentration in solution could be reduced by adsorption to the clay particle to an extent sufficient to preclude or greatly reduce the rate of decomposition. In this latter situation, strong binding of the carbonaceous substance to the clay particle reduces the probability of its collision with and uptake by the degrader population. The rate of desorption from the clay thus becomes the limiting factor determining the rate of decomposition of the carbonaceous energy source. Thus seemingly contradictory observations as suggested above could be reasonably explained by examining the chemical processes associated in the partitioning of the carbon and energy source between the bound and soluble nutrient pools and the effect of proportion of the organic substances in the soil that is bioavailable.

Thus, the impact of clay sorption in soil ecosystems is more a function of residual concentrations of organic compound in interstitial waters and in the microsite containing the microbial colony than is the apparent concentration when the bulk soil sample is analyzed (i.e. microsite concentrations of the compound of interest may far exceed macrosite levels). Thus, decomposition kinetics of the substance reflect the microsite concentrations rather than those that would be predicted from total ecosystem content of the carbonaceous growth substance.

Not only do clay minerals alter decomposition kinetics of organic substances in soil through sorptive-desorption reactions, but they may also indirectly affect microbial metabolism by altering their chemical environment. For example, due to the formation of an hydrogen ion cloud around the clay particle, the pH of the microsite is reduced (see Figure 1.7). Thus, enzyme-catalyzed reactions in clay soils may appear to have pH optima lower than that detected in the absence of clay particles.

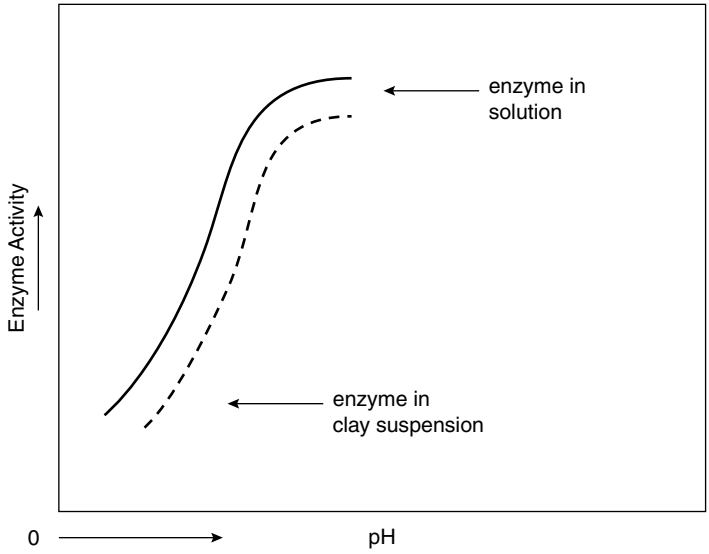

Figure 1.7 Hypothetical data reflecting alteration of apparent pH optima by microsite variation in pH due to clay micelles.

1.2.1.2 Humic Substances and Ecosystem Function

Soil organic matter is commonly considered to be equivalent to soil humus (Stevenson 1994). The soil humus fraction consists of all organic compounds normally found in soil (with the exception of recognizable plant or animal components), their partial decomposition products, and microbial biomass. Studies of the nature of soil organic matter are complicated by the diversity of materials present and the fact that the definition does not coincide with what can be reasonably separated and analyzed; for example, clear separation of plant and animal biochemicals from the bulk soil organic matter is not easily accomplished. The mixture of chemical substituents of soil humus ranges from simple monomeric components of living cells, such as organic acids, amino acids, and saccharides, to the random polymers classified as humic acids. Essentially any organic compound contained in or synthesized by living cells occurs in soil, although some may be present in minuscule amounts due to their being relatively easily decomposed by soil microbes.

Soil humic substances: The conclusion that soil humic substances have a major input in determining the fundamental soil properties demands a deeper consideration of the basic properties of these substances. By definition, humic substances are a series of high molecular-weight, brown to black substances formed by secondary synthesis reactions in soil. This organic matter fraction is not designated or isolated from soil based on a function or role in the soil ecosystem. It is a portion of

soil humus that is defined strictly by its solubility in acidic or alkaline solutions. Humic substances are separated from soil with alkaline solutions. Acidification of the alkaline extract to a pH between 1 and 2 results in precipitation of the humic acids. Fulvic acids are soluble in both acid and alkali. Humin is that portion of the colloidal soil organic matter fraction that is not solubilized with alkaline solutions

To understand the impact of humic substances, especially humic acids, on microbial function in the soil ecosystem, an appreciation of the structural components interacting with soil particles and the microbial community is necessary. Humic and fulvic acids are heterogeneous, random chemical associations of a variety of aromatic and aliphatic components. No single chemical structure can be proposed to represent these molecules. Indeed, because a wide diversity of aromatic and aliphatic compounds are linked via a variety of covalent linkages forming humic acids with molecular weights in the thousands of daltons, it can reasonably be concluded that few if any of the molecules are identical. In contrast, fulvic acids are more oxidized than humic acids; that is, they have higher oxygen content and a lower carbon content and molecular weight.

Structural implications of humic acids: The nature of the constituents of humic substances determines their importance to total ecosystem function. The heterogeneity and complexity of humic and fulvic acids have limited the level of understanding of the specific chemical reactions between humic and nonhumic soil organic components. An appreciation of the types of reactions occurring between microbial cells or individual biochemicals and humic acids has developed from our basic concept of the molecular content of humic substances and the use of related model compounds.

Our concept of humic acids structure and *in situ* interactions in soil was improved through the application of spectroscopic analytical techniques, such as NMR spectroscopy. (See Preston (1996) for a review of the utility and limitations of NMR techniques in elucidating the elements of humic acid structure.) Data derived from NMR-based analytical procedures suggest that aromatic rings make up a smaller portion of the humic acid molecule than was previously believed (e.g. see Hatcher et al. 1981; Malcolm 1990). Hatcher et al. (1981) found that aromaticities of humic acids from a variety of soils collected from Prince Edward Island ranged from 35% to 92%. Significant aliphatic structures were generally noted. These were not detected by chemical oxidation procedures. These authors also suggested that phenolic carbons may be minor components of humic acids. They were previously considered to be major substituents. This conclusion was based on the observation that NMR spectra did not contain intense signals corresponding to the presence of these molecules. Schnitzer and Preston (1986) suggest that since the presence of phenolic hydroxyls is confirmed by several independent methods, failure to detect them by NMR analysis merely indicates a shift in the NMR signal resulting from these moieties.

With the development of more sensitive methods for analysis of humic acid structure, a clearer understanding of the interactions of these substances with soil minerals as well as xenobiotic chemicals is becoming possible (for more information on this topic refer to Cao et al. 2011; Simpson 2006). Particularly of interest in determining the capability of xenobiotic chemicals to be sequestered within humic acids is the debate regarding the total size of the humic acid molecules and whether they are composed of aggregates of smaller molecules, single very large molecular weight molecules, or a combination of both (e.g. see Cao et al. 2011; Li et al. 2003; Piccolo et al. 2002).

The primary conclusion regarding the structure of humic substances and their importance to the soil microbiologist is derived from the complexity of their composition. They contain varying ratios of phenolic, methoxyl, aromatic, hydrocarbon, amino acid, and nitrogen moieties plus covalently linked polysaccharides and proteins. The ratio of these substituents is dependent on the source of the humic acid. Great variation in composition occurs between humic acids prepared from soil, stream, and marine samples (Malcolm 1990). Significant differences in humic acids have also been noted for humic acids prepared from a variety of soil types – alfisols and mollisols (Novak and Smeck 1991) – and soil management histories (Stearman et al. 1989).

Humic substances, organic compounds, and biodecomposition: Although a variety of microorganisms have been suggested to be capable of metabolizing humic acids, it is unlikely that humic acids are meaningful contributors to the energy resources of soil microbes. This conclusion results primarily from observations of the complexity of the structure of molecules – they contain a large variety of substituents that are essentially randomly assembled. Therefore, an inordinate amount of metabolic energy would have to be expended in synthesizing the large array of degradative enzymes needed to decompose the humic acids – considerably more energy than the microbe would recover from mineralization of the complex molecule. Humic substances affect soil biological processes to the greatest extent by interacting with soil minerals in soil aggregate formation (see Section 1.2.2) and by their role in removal of biodegradable substances from the pool of easily metabolized substrates (i.e. humification). Enzymatic interaction with humified substrates is inhibited physically by the imposing humic acid physical structure.

As was introduced above in the discussion of clay/organic substance interactions, humic substances sorb organic compounds through temporary interactions. For these organic matter interactions, sorption results from *n–n* interactions, van der Waals forces, and hydrogen bonding as well as ionic associations. More permanent linkage of organic compounds to humic substances also occurs due to formation of covalent bonds. Examples of organic compounds that may be linked covalently to humic acids include amino acids, peptides, proteins, aromatic compounds, and polysaccharides.

Covalent linkage of phenolic compounds to humic acids may be catalyzed by phenoloxidase (Bollag et al. 1980; Dec and Bollag 1997; Liu et al. 1985). Carbon–carbon and carbon–nitrogen linkages are catalyzed through free radical formation by these laccase-type enzymes (Figure 1.8). The linkage of aromatic ring-containing compounds occurs in *ortho-ortho* or *para–para* or *ortho-para* type associations by this biologically catalyzed process (Sjoblad and Bollag 1981). Aside from being a mechanism for the formation of humic substances *in situ,* these oxidative processes provide a means for the stabilization of a variety of xenobiotic compounds, such as pesticides, into soil organic matter.

The impact of the covalent association of organic compounds with humic substances on their biodecomposition kinetics is more predictable than was described above for clay–organic matter interactions. Humification of organic

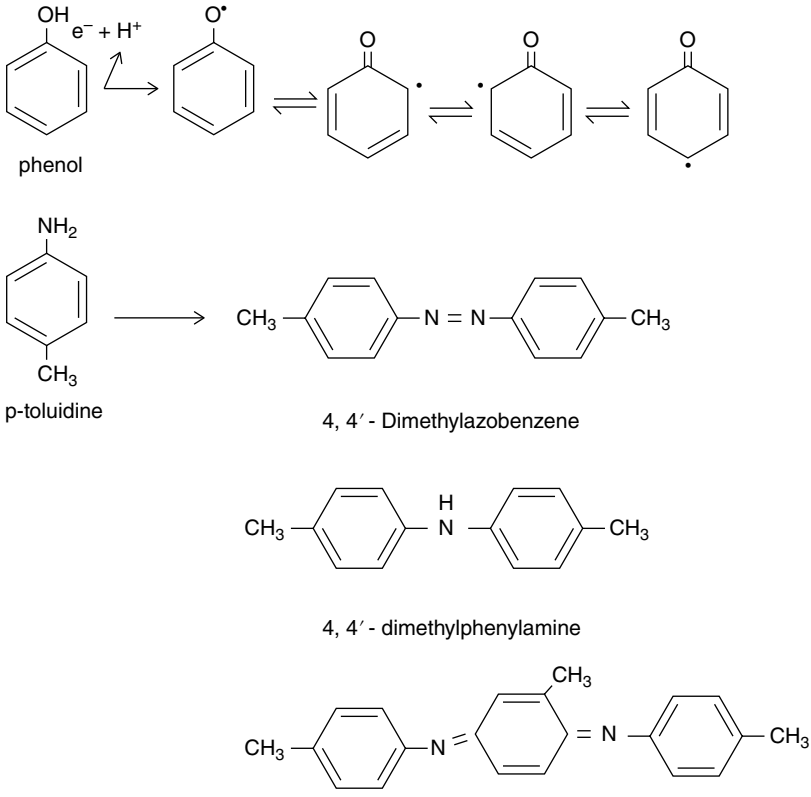

Figure 1.8 Oxidative coupling of aromatic compounds. Mechanism showing formation of free radicals and examples of some products of *p*-toluidine polymerization. See Sjoblad and Bollag (1981) for further discussion and examples.

compounds results in their stabilization in soil. For example, in a study of the impact on microbial degradation by nucleophilic addition of amino groups to the aromatic nuclei, Bondietti et al. (1972) found that bonding of amino sugar units to model humic acids resulted in major reductions in the decomposition rate. Over a 12-week incubation period, more than 70% of free glucosamine was oxidized to carbon dioxide, while only 15–23% of the humic acid-model polymer-bound amino sugar was oxidized. The importance of covalent linkage of the humic acids and sugar was shown by the fact that simply mixing the amino sugar with the model humic acid molecule did not reduce the oxidation rate.

It is important to note that substances covalently bonded to humic acid can still be mineralized but that the rate of oxidation is much slower than for nonchemically linked substances. Basically, what is under consideration here is the fate of the carbon of the humified organic compound after it has been incorporated into the humic acid molecule. Since it is part of the larger substance – that is, humic acid – its decomposition kinetics tend to resemble the slow mineralization rate of humic acid molecules. This observation has environmental significance. Covalent bonding to humic acids could be a viable mechanism for renovation of xenobiotic compound-contaminated soils if the substances are permanently bound or if they are released in an inactivated (i.e. partially or totally catabolized) form. Unfortunately, this condition is not always met. Release of humic acid-bound pesticides intact can occur (Bartha 1980; Lichtenstein 1980; Still et al. 1980). Thus, from an agricultural viewpoint, difficulties may arise from release of a pesticide that had been previously applied to an appropriate crop and stabilized in soil humic substances several years after application. Particular concern would arise should this pesticide reappear in a crop where use would be inappropriate from either a crop sensitivity or regulatory view. Additionally, incorporation of photosynthetically fixed carbon into humic substances and associated molecules mitigates at least a portion of the impact of carbon dioxide production on global climate change.

It must be stressed that in most cases, the more probable fate of a biodecomposable substance added to soil is mineralization, either before humification or even subsequent to humification. For example, Haider and Martin (1988) found that *Phanerochaete chsysosporium* could mineralize xenobiotic compounds at a reasonable rate (conversion of 13–56% of xenobiotic carbon as carbon dioxide over an 18-day period). Similarly, Saxena and Bartha (1983a) found that humus-bound 3,4-dichloroaniline (a biodegradation intermediate) was mineralized at a rate approximately the same as that of the humic acid molecule itself. Adsorbed 3,4-dichloroaniline was mineralized faster than humic acid molecules. Of note is the observation that fertilization of agricultural soil with anhydrous ammonia could mobilize humus-bound 3,4-dichloroaniline (Saxena and Bartha 1983b).

The size and complexity of the humic acid molecule create the potential for a stable, noncovalent association (sequestration) between it and simple organic

molecules. Using the classic formula for the humic acid molecule, Schulten and Schnitzer (1997) have described a three-dimensional structure of the molecule that can accommodate noncovalent associations of simple organic molecules within voids in the molecule. These associations are sufficiently stable energetically that the longevity of the associations between the simple and complex organic substances resembles that of covalently associated molecules (Dec and Bollag 1997). Hydrophobic compounds have been shown to associate with hydrophobic domains, micelles, within the humic acid molecule (von Wandruszka 1998). Such associations may participate in reduction of bioavailability of toxicants through sequestration (see Chapter 16). Environmental concerns relating to these noncovalent associations relate to the need to assess the extent of their occurrence in native soil ecosystems, to evaluate the potential for release of the toxicant from the association, and to quantify the environmental risk resulting from the freeing of the toxicant from the humic acid mycelle.

1.2.2 Aboveground and Belowground Communities and Soil Ecosystem Synergistic Development

A primary controller of the structural associations of soil minerals is the interactions between soil microbes, higher plants, and soil particles. These interactions in turn control total ecosystem development, sustainability, and quality. A visual examination of a productive surface soil easily leads to the conclusion that the sand, silt, and clay components tend to be aggregated into larger associations – soil aggregates. Optimization of the soil aggregate structure produces a soil environment more supportive of plant growth. Thus, the question arises of how soil aggregates are formed.

A somewhat cursory consideration of the basic interactions between soil microbes, higher plants, and soil particles provides a foundation for understanding how these more productive structures come to be. The first consideration is that the soil microbial community derives the bulk of its cellular energy from the oxidation of fixed carbon produced via plant photosynthesis. Layered upon this fact is the observation that maximal plant biomass production relies in part on existence of a soil physical structure conducive for plant growth. The microbial community is a primary determinant controlling optimization of the soil structure for plant growth. For example, the soil structure must be such that water infiltration and oxygen diffusion are not impeded as is exemplified in the extreme situation by a compacted soil. In such a soil, soil quality and therefore plant biomass productivity are limited. This conclusion is predicated on the understanding that the evolution, growth, and sustainability of the plant community are determined by interactions of the soil microbial community with soil particulate matter. Soil bacteria and fungi are major players in the grouping of soil particulates into aggregates. A plant

growing in a poorly aggregated soil provides only a limited amount of the carbon and energy required for sufficient microbial productivity to optimize soil aggregate structure. But, even in low productive ecosystems some carbon and energy are provided to the soil microbes by the limited plant growth; that is, sufficient energy to increase the level of formation soil aggregates at least minimally. The small improvement in soil structure could subsequently increase the amount of microbial biomass, so in turn if the soil is properly managed this results in improvement of the soil structure, which can allow for an increase in the ecosystem productivity. Two such systems – soil aggregate formation and biological soil crusts – exemplify how soil ecosystems are built upon these synergistic interactions.

1.2.2.1 Soil Aggregate Structure Development and Its Impact on Ecosystem Function

The world of the soil microbe does not consist of individual sand, silt, or clay particles or even of organic matter *in toto*. The microbial community must be capable of interacting with a mixture of these substances assembled in a seemingly random order. Yet when examined microscopically, the soil microorganisms may appear to be associated with one specific soil component in this complex environment. For example, Fisk et al. (1999), using a combination of specific stain and micromorphological techniques, found that soil-amended bacteria tended to become associated with variably charged mineral oxides and organic matter within the intergrain microaggregates of a Freehold sandy loam and occurred along coated mineral surfaces. Also, rhizosphere organisms may be growing essentially on root surfaces. Microbes may be physically attached to partially decomposed plant debris. But even in these situations, the metabolism of soil microbes is affected by the association of the soil particle to which they are attached within larger soil aggregates. Note that the soil particles, the microbial community and the plant root system interact to provide a structure with enhanced water infiltration and retention, encourage the cycling of plant carbon, nitrogen, and phosphorus between belowground and aboveground communities, and facilitate optimization of a soil structure that supports ecosystem productivity and sustainability.

Soil structure is defined at the level of the microbial cell by the association of soil particulates and colloidal organic matter into soil aggregates. As noted previously, soil aggregates are combinations of soil organic and mineral components assembled into microaggregates (<50 μm mean diameter) and macroaggregates (>50 g mean diameter particles).

Soil aggregate formation: Assembly of soil particulates into aggregates is an excellent example of biotic and abiontic organic matter plus living cells and mineral components interacting to affect soil physical properties. The biological contributions to soil aggregate formation can be described as a coupling of feedback-controlled processes (Figure 1.9). Biological participants are higher plants as well

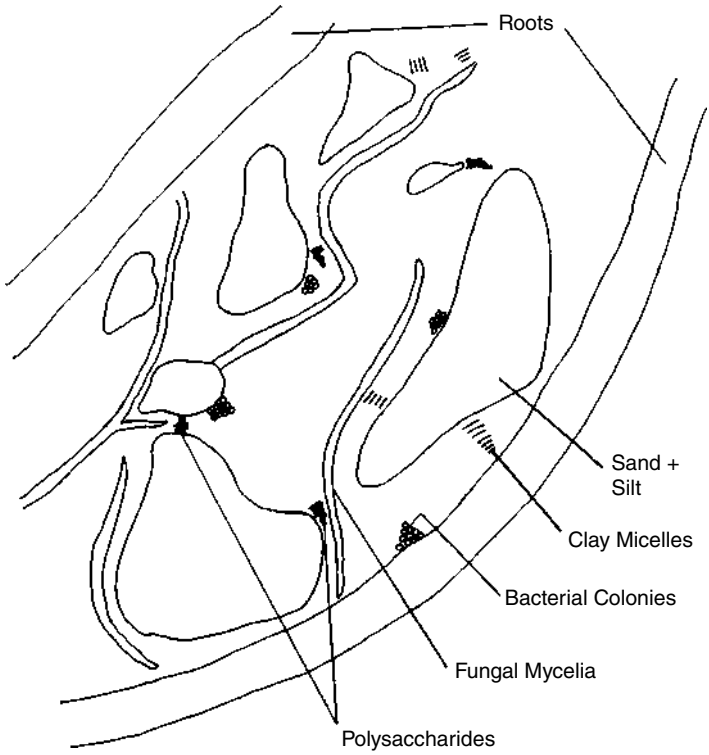

Figure 1.9 Basic elements of soil substituents contributing to aggregate formation (not drawn to scale).

as soil microbes. The soil physical matrix is produced through the synergistic interactions of the biological entities associated with the soil particulates. Microorganisms metabolize photosynthetically fixed carbon to produce the compounds responsible for binding soil mineral matter together; that is, polysaccharides and humic substances. Humic acids are the product of aliphatic and aromatic compounds, some of which are biologically synthesized, being chemically and biologically linked into complex high molecular weight compounds. Improvement in the size and number of aggregates formed enhances plant community growth, which in turn stimulates even further microbial development by producing increased levels of fixed carbon. Soil quality and associated functions are enhanced in proportion to the degree of soil aggregation. Note that aboveground plant community development can be enhanced directly in proportion to the soil aggregate structure improvement depending upon the inherent production capacity of each species. In contrast, poor soil structure retards air diffusion, water movement and root growth, thereby resulting in a less active soil microbial community than would

be attained under normal optimal growth conditions. Recall that mineralization of the plant biomass is the primary energy source for the soil microbes. Thus poorly aggregated soil would result in limiting fixed carbon inputs into the soil. As soil aggregate stricture is improved, the production of plant biomass allows for enhanced microbial biomass production. Additionally, optimization of the microbial decomposition of the fixed carbon improves the recycling of nutrients such as nitrogen back to the growing plants.

A classic conceptual model presented by Tisdall and Oades (1982) provides insight into the role of the biological community in aggregate formation and a basis for proposing a model of how aggregate formation represents a synergistic interaction between aboveground and belowground life forms. In the model, organic binding agents are divided into three categories: transient (mainly polysaccharides), temporary (roots and fungal hyphae), and persistent (humic substances associated with polyvalent metal cations). Soil aggregates are formed in a hierarchical arrangement by stabilization of smaller aggregates into larger structures by different binding agents (Waters and Oades 1991). Microaggregates are stabilized by persistent binding agents, whereas polysaccharides and biomass (microbial and root) result in the less persistent association of the microaggregates into macroaggregates (Beare et al. 1997; Elliott 1986; Hu et al. 1995). Microaggregates consist primarily of clay and humified organic materials linked by polyvalent metals (Edwards and Bremner 1967). Due to the biodegradation resistance of the persistent binding agents, the microaggregate structure is long term and appears to be more a characteristic of the soil than a product of soil management procedures. In contrast, the assemblage of microaggregates into macroaggregates is less stable and affected more by soil management due to its reliance on soil fungal and root development, including mycorrhizal associations (Hu et al. 1995). This model emphasizes the importance of polysaccharides and humic substances in soil aggregate formation, but it must be appreciated that any organic compound that stimulates or decreases microbial growth in soil indirectly affects aggregate formation by affecting the growth dynamics of the fungal community.

As could reasonably be anticipated by the high variability of soil mineral components and organic matter, the complexity of the micro- and macroaggregate structure is more complex than implied by the Tisdall and Oades (1982) model. For example, Watteau et al. (2012) demonstrated that associated with the variable complexity and composition of soil mineral matter comes an increased complexity of classes of microaggregates. The quantity of the organic matter entering the soil affects the dynamics of aggregate formation and stability. For example, more easily decomposable organic residues tend to result in a rapid transient increase in aggregates whereas more slowly mineralized organic matter results in a slower but longer lasting response to the increased energy supply to the soil microbial community, as exemplified by research of Le Guillou et al. (2012). Organic matter

quality and its nitrogen content also affect the dynamics of aggregate formation and stability (for example, see Le Guillou et al. 2012 and Gude et al. 2012). Note that the impact of organic matter on this important aspect of soil structure results from turnover of organic matter produced by the native plant community as well as the impacts associated with anthropogenic management of the soil.

Role of carbohydrates in soil aggregate formation: The importance of polysaccharides as binding agents for adhesion of soil mineral components into aggregates has long been appreciated. Early research involved correlation of polysaccharide levels with quantities of water-stable aggregates, quantifying the effect of periodate oxidation of polysaccharides on aggregation, and analyzing the influence of polysaccharide adsorption on the mechanical strength of clay minerals. For example, Chaney and Swift (1984) found that total organic matter, total carbohydrate, and humic material exhibited high or very high significant correlations with aggregate stability in 120 soils. To rule out other soil physical properties as effectors of aggregate formation, 26 agricultural soils were evaluated. Only organic matter and associated properties correlated significantly with aggregate stability. Other soil parameters examined were sand, silt, clay, iron content, and cation exchange capacity.

As noted above, direct evidence for the role of polysaccharides in soil has been gained by assessing the effect of their selective destruction on soil aggregation. Periodate treatment of soil results in oxidation of polysaccharides contained therein. Not all polysaccharides are destroyed by such treatment. The proportion of polysaccharides oxidized depends on the duration of the treatment and the extent of contact of the polysacharide with the oxidizing agent. The polysaccharide may be occluded by close association within soil particulate structures. Even with these limitations, a major role of polysaccharides in aggregate formation is revealed by this method. Periodate treatment of soil from a long-term grass field destroyed microaggregates greater than 45 μm in diameter along with the carbohydrates (Cheshire et al. 1983). Residual carbohydrate was enriched in sugars commonly found in plant materials, suggesting preferential destruction of microbial polysaccharides by the periodate. The relationship between soil polysaccharide and aggregate stability was verified in 15 soils and seven soil series (Cheshire et al. 1984).

Chenu and Guerif (1991) assessed the strength of the association between polysaccharides by assessing the mechanical strength of mixtures of kaolinite or montmorillonite plus or minus the polysaccharide scleroglucan. The polysaccharide increased the mechanical strength in mixtures of both clays through the formation of polymer bridges between the clay particles and from the progressive coating of the clay surface by the polysaccharide. The primary effect of the polysaccharide on soil aggregation was therefore the result of increased strength of interparticle bonds.

Humic acids and aggregate stability: Microaggregate formation results from interactions between humic substances and clay particles (for example, see

Capriel et al. 1990; Chefetz et al. 2002; Dinel et al. 1991; Schnitzer and Kodema 1992). Aliphatic side chains are particularly instrumental in this process. A high correlation between soil aliphatic fractions (Capriel et al. 1990) and aliphatic-enriched humic fractions (Dinel et al. 1991) with aggregate stability underscores the importance of these fractions in microaggregate formation. As was noted above, humic acid-associated aliphatics can form interlayer associations with clays (Theng et al. 1986), which are enriched in soil clay fractions (Arshad and Lowe 1966; Schnitzer et al. 1988). An early study by Schnitzer et al. (1988) demonstrated that although the quantities of aliphatic fraction extracted from soil with supercritical-hexane varied with management, the chemical properties revealed by ^{13}C NMR and Fourier-transform infrared differences were independent of management (Schnitzer et al. 1988).

Soil management and aggregate stability: The stability of microaggregates and macroaggregates reflects the biodegradation susceptibility of the primary organic polymers or biological biomass instrumental in their formation. Microaggregates stabilized through association of humic substances with clay particles are more resistant to management procedures and are more reflective of soil physical properties.

Soil management typically results in modification of factors controlling soil microbial activity. For example, cultivation of soil can enhance the degradation of organic matter previously occluded by soil minerals, increases in soil aeration, and modification of soil pH through liming. Portions of the soil organic fractions could be depleted under appropriate conditions in a matter of days or weeks. Therefore, soil management procedures that cause a decrease in soil organic components result in a reduction of quantities of the water-stable macroaggregates produced by their interaction with soil particulates. Examples of declines in soil structural properties are found in studies of the effect of long-term cultivation on soil properties. For example, Tiessen and Stewart (1983) evaluated the effect of cultivation for between four and 90 years on soil particle size distribution of three North American grassland soils (Cryoborolls). They noted the continuous depletion of organic matter and the loss of particles $>50\,\mu m$ diameter with time of cultivation, suggesting that the more degradable metabolizable soil organic matter components supporting the structure of soil aggregates were being depleted with time.

Conversely, soil management procedures chosen to increase microbial activity, resulting in an increase in biomass and polysaccharide synthesis, cause improvements in soil aggregate structure, as was reflected in the study of Tisdall and Oades (1982), where the duration and magnitude of the improvement in soil microstructure were shown to be dependent on the nature of the metabolized carbon substrate added to the soil samples. Maximum aggregation occurred in soil amended with the rapidly metabolized substrate, for example glucose, whereas the more lasting but less dramatic improvement in soil structure was detected with more slowly metabolized cellulose. Although cellulose is much more biodegradation

resistant than is glucose (months compared to days for depletion from soil, respectively), each of the materials could be considered to be rapidly catabolized when compared with humic substances (degradation times measured in decades or more). Reduction of tillage intervention with crop production is another example of a means of manipulating soil communities and organic matter levels in order to improve aggregate structure. Changing from an intensive cultivation management strategy to no-till or minimum-till operation results in increases of soil humus levels and augmented microbial biomass, which coincides with improvements in the soil aggregate structure.

The impact of carbon inputs from crop growth on aggregate formation also can vary with the species grown. For example, Angers (1992), in a comparison of the effect of alfalfa (*Medicago sativa* L.) or corn (*Zea mays* L.) growth on the aggregate structure of soils from bare fields, found maximum aggregation under alfalfa with minimal changes in this soil property during the study in the fallow field and that cropped to corn. Most likely, the effect on soil aggregation resulted from a combination of variation in quantities and quality of organic matter available to the soil microbial community plus the resultant alteration of the activity of these populations. This effect of plant growth of soil aggregate structure provides an excellent example of a situation where the aboveground community and soil microbes interact to improve the quality of a degraded soil.

Intensive agriculture resulted in major reductions in the soil organic matter reservoir with a parallel reduction in soil quality and crop yields, thereby having significant impacts on associated communities and societal development (e.g. see French 2010; Kareiva et al. 2007). Appropriate cropping practices can increase soil aggregation and optimize soil microbial activity and crop yields. The increased formation of soil aggregates results in better soil quality, allowing for improved plant growth. With increasing plant biomass production as soil quality improves, more organic matter is provided to the microbes which results in optimization of activity and productivity of both aboveground and belowground communities. Additional stimulation of the soil organic matter pool and associated improvements in soil quality can also be encouraged by recycling of societal organic waste materials. The effect of such amendment on soil aggregate formation can be substantial. For example, Glauser et al. (1988) found an increase of water-stable aggregates to 85% on soils receiving biosolids amendments, compared to 45% in nonamended soils.

1.3 The Macro-ecosystem

An appreciation of variation in soil properties on a macro-scale is equally important to understanding the microsite characteristics described above. Both the vertical and horizontal variation in soil properties limiting microbial activity must be quantified.

Although a considerable interest is developing in estimating microbial function in deep aquifer materials, most soil microbiology research has been aimed at developing an understanding of processes occurring at the point of contact between the aboveground and belowground communities. Thus, soils from the 0, A, and to some extent the upper portions of the B horizon are most frequently studied. Interactions in the B horizon are especially important in sites where the 0 horizon is nonexistent and the A horizon is thin or has been lost through erosion.

The importance of these major surface soil horizons is derived from the fact that the primary energy source for the soil microbial community is photosynthate entering soil primarily as plant biomass or root exudates. Although some water-soluble plant-fixed carbon may leach through the soil profile, especially in soils with measurable organic horizons, the primary microbial encounter with organic carbon-containing materials is in the top few centimeters of the soil profile. Thus, a major concern in assessing the microbial community is the distribution of carbon inputs on the soil surface – that is, plant community growth and dispersion of fixed carbon within the soil profile due to past biomass productivity and its transformation and redistribution within the soil matrix. Secondary considerations relate to variation of physical and chemical growth affecting parameters across the soil surface and throughout the profile. Again, many of these result from activity of the aboveground community. Other factors that must be considered are temperature (which varies diurnally as well as seasonally and is affected by plant density and degree of canopy closure) and moisture (which may also vary seasonally and is affected by topography and plant canopy development). Distribution and amount of microbial activity in soil horizons can also be related to soil parent material and soil developmental history as well as current properties such as pH and salinity. Distribution and range of variation of each of these factors must be considered when extrapolating microbiological data to the ecosystem level.

Variation in soil microbial activity in surface soils is generally controlled by inputs of energy in the form of photosynthetically fixed carbon contained in or exuded from plant biomass. The impact of this energy supply distribution is exemplified by examining the sources of fixed carbon in a temperate forest soil. Within the forest soil, microbial energy is derived from oxidation of fixed carbon contained in litter and from decomposition of root exudates, sloughed cells, and fine roots. The contribution of fine roots to the soil nutrient pool is frequently overlooked, but it can constitute a major source of nutrients and energy for soil microbes (see McClaugherty et al. 1982, 1984; Vogt et al. 1983 for examples). In two temperate forest stands, fine root production was roughly equivalent to leaf biomass production (McClaugherty et al. 1982). If an O horizon has developed, microbial activity in the A horizon is also influenced by fixed carbon carried in leachates and by that portion of the plant biomass mixed into the soil surface by earthworm and other animal activity. Thus, maximal soil microbial activity would

be anticipated to occur in the vicinity of the tree or shrub (and grass if it occurs on the forest floor) and under areas of litter accumulation and decomposition.

A similar distribution pattern of plant biomass is observed in a grassland soil. Grass biomass enters soils as leafy material in the thatch and as root exudates and decaying root biomass in a dense root structure. Higher concentrations of water-stable aggregates are generally found in soils collected from grassland ecosystems compared to soils from other major ecosystem types, if all other limiting factors are equivalent. This results from the high input of biomass, the consistent production of this fixed carbon throughout the growing season, and the proportion of readily decomposable components constituting the plant debris as compared to alternate ecosystem types such as forest or agricultural soils. For example, Jenkinson and Ayanaba (1977) noted that 70% of ryegrass (*Lolium multiflora*) decomposed with a half-life of 0.25 years in agricultural soils of Rothamsted, England. The remainder had a half-life of eight years.

With agricultural soils, the organic matter inputs are commonly similar to those of the grassland in quality, but their distribution and quantities are more limited than is observed with the grassland. Thus, soil structural development is reduced. In intensively cultivated sites, reduction of colloidal organic matter levels in the soil results in a loss of soil microstructure (see Tate 1987 for further analysis of limitations and soil structure in this ecosystem type). Analysis of microbial activity in these soils must be conducted with an understanding of crop planting patterns, cropping history, and tillage practices. In widely spaced grass crops, such as sugarcane (*Saccharum* spp. L.), biomass inputs to the microbial community between rows are minimal.

Organic matter generally accumulates in swamp ecosystems. Organic matter inputs from the aboveground plant community are limited by the high soil water table, as is the decomposition rate of the decaying plant biomass. The accretion of organic matter indicates that biomass production occurs more rapidly than its decomposition. Other organic inputs to the swamp system originate from erosion of soils. Since the A horizon soils contain most of the colloidal soil organic matter, washing of these soils down slope results in importing of this organic matter into the swamp environment.

1.4 Concluding Comments

The act of allowing a soil sample to flow between one's fingers does not leave the impression that a living, respiring community is functioning therein. It could rather easily be concluded that the utmost importance in the various soil types must be attributed to their physical components – that is, their sand, silt, clay, and, perhaps, rock and pebble contents. Although such a lack of apparent importance

of biological entities is at first disappointing, such an observation is critical for the development of our concepts of soil microbiology. It is too easy for soil microbiologists to concentrate on the extremely diverse community of microbes existing in a soil sample and their vast array of genotypic and phenotypic properties, and to forget that these organisms are of no benefit to the total ecosystem if they are incapable of functioning within the soil physical matrix. The physical and chemical properties of soils and their variability (macro- and microsite) must always be appreciated if a true understanding of the limitations and capabilities of the soil microbial community is to be fully developed.

The variability of the soil environment and its impact on life processes are both intriguing and frustrating. As microbiologists are keenly aware of the fact that no typical microbe exists for even the simplest physiological or ecological study, they must also appreciate the fact that there is no standard soil. Soil components and their interactions are at least as variable as are the characteristics of their associated microbial populations. Thus, a complete understanding of soil microbial behavior and its contributions to total ecosystem function must include an appreciation of its physical environment – both the mix of individual soil components as well as the physical impediments presented to the soil microbes by the assembly of these discrete particles into a defined soil structure, including horizonal development as well as aggregate formation. Assessment of the soil type and its management history is as important to the conducting of "good science" as is provision of a genus or species name to the microbes studied. That is, a complete soil microbiological study must result not only in an appreciation of the nuances of the biological aspects of the soil ecosystems, but also in a conceptualization of the impact of variations in the physical and chemical environment on the expression of the living capacities.

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2

The Soil Ecosystem

Biological Participants

Soils may contain a variety of easily visible and identifiable life forms, including plant roots, insects, and earthworms. If these macroorganisms are removed, the remaining bulk soil may appear to the casual observer to be a lifeless mass. But this seemingly lifeless mass of clay, sand, and silt, which easily slips between our fingers, is home to an incredibly diverse and complex community of organisms that are too small to be seen with the unaided eye, including bacteria, archaea, fungi, protozoa, and viruses. This microbial community accounts for less than 0.5% (w/w) of the soil mass yet, these microorganisms have a major impact on soil properties and processes. Indeed, the integrity of the total ecosystem, both above and below ground, rests on the stability, resilience, and function of the soil microbial community. Destruction of the soil microbiota through mismanagement or environmental pollution can result in decline or even death of the aboveground plant and animal populations. Thus, development of an understanding of soil microbes, their properties, and the nature of their interactions with and within their environment is essential.

The study of soil microbial communities presents some unique challenges, due to factors such as their small size, incredible diversity, and heterogeneous distribution, as well as the heterogeneous nature of soils themselves (as discussed in Chapter 1). The objective of this chapter is to provide an overview of some key characteristics of soil microbial communities and their relationship to total ecosystem function. This discussion of soil microbes will include a presentation of some commonly used analytical procedures available for quantifying soil microbial populations.

2.1 The Living Soil Component

Historically, research on soil microbial communities has come from three main perspectives: (i) identification of the organisms present and elucidation of their relationships with other members of the soil community, i.e. basic community

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ecology studies; (ii) assessment of the activity of microbes participating in processes of agricultural importance, such as nitrogen fixation, nitrogen mineralization, denitrification, or pesticide decomposition, in order to inform agricultural management practices; and (iii) isolation of organisms capable of producing anthropogenically important products, e.g. antibiotics. More recently, with the observation that human activities have caused a decline in the quality of soil resources, considerable effort has been expended in the study of soil microbial processes associated with maintaining ecosystem stability or renovation of damaged sites. For example, there has been great interest in determining the identity and physiological properties of microbes involved in the degradation of a variety of toxic compounds, with the goal of developing bioremediation strategies.

Since soil bacterial and fungal communities are prime participants in each of these areas, most research on soil microbiology has focused on these two groups of organisms. Thus, much of what is described in this chapter relates to these two groups, yet it must be remembered that interactions with protozoa, nematodes, and other higher life forms help to maintain the vigor and productivity of the bacterial and fungal communities. Chapter 6 provides a more detailed discussion of these interactions. In addition, there is growing evidence that archaea, a group of prokaryotes once thought to be mainly extremophiles, are more numerous in soils and play a more significant role in soil processes than was previously recognized. For example, the recent discovery of ammonia-oxidizing archaea (Könneke et al. 2005) and the demonstration that these organisms outnumber ammonia oxidizing bacteria in a variety of soils (Leininger et al. 2006) suggest that archaea may be significant contributors to ammonia oxidation in soil, a process that was previously assigned exclusively to the bacteria.

2.1.1 Biological and Genetic Implications of Occurrence of Living Cells in Soil

To gain a full appreciation of the consequences of biological activities in soil, several unique properties of the living component of soil must be examined in greater detail. Characteristics of soil microorganisms that confer special properties to soil processes include that (i) they possess a diverse metabolic capacity encoded in their genomes that is not necessarily expressed phenotypically at any particular point in time, (ii) they replicate, (iii) they have a definite cellular structure, (iv) they form resting structures, (v) they catalyze reactions that change the soil, and (vi) they are a link between aboveground and belowground processes.

2.1.1.1 Gene Pool Potential

A unique property of the soil microbiota is that the biochemical potential contained therein is significantly greater than that which is expressed at any particular point in

time. There are several reasons for this phenomenon. First, many of the genes within the genomes of active soil microbes are not expressed under a given set of environmental conditions. Second, many microorganisms in soil are dormant; that is, they are present in an inactive resting state, and essentially none of the products of the genes contained in these inactive microbes is produced (Lennon and Jones 2011). Finally, advances in sequencing technology have revealed that soil microbial communities include a vast number of taxa that are present at very low abundance, a pool of organisms that has recently been termed the "rare biosphere" (Sogin et al. 2006).

Research on the rare biosphere is still in the early stages, and one question that remains unresolved is whether these rare taxa are dormant or active but present in very low abundance, perhaps due to very slow metabolic rates. Recent studies have indicated that it is possible for some rare taxa to multiply and become dominant under appropriate conditions, a phenomenon termed "conditionally rare taxa" (Shade et al. 2014). Therefore, it can be concluded that three major pools of potential but inactive metabolic processes found in soil are (i) active microbial cells with noninduced enzymatic capability, (ii) cells present in an inactive or dormant state, or (iii) cells present with insufficient population densities to produce measurable effects on soil metabolic activity, i.e. the rare biosphere. Based on these scenarios, we can conclude that genotypic traits exist within the soil microbial biomass which when they are induced could result in a biological community with entirely different properties than currently exist.

Induction of metabolic activities in soils: Synthesis of requisite enzymes for previously unexpressed metabolic activities is induced primarily by changes in the physical or chemical properties of the soil. Physical modification of the soil properties can be exemplified by the development of anoxic conditions. Imposition of this oxygen-free status commonly occurs as the result of flooding where the excess soil moisture is retained under reasonably static conditions. The lack of water movement within and overlying the soil limits the transport of oxygen to soil microsites. The only source of molecular oxygen becomes diffusion from the atmosphere through the water into soil pores – an extremely slow process. This situation coupled with an actively respiring aerobic microbial community results in rapid depletion of free oxygen in water-saturated soils. Prior to exhaustion of the free oxygen supplies, aerobic metabolism predominates. Without a continued oxygen supply, aerobic respiration cannot continue and enzymes associated with anaerobic respiration are induced.

One example of a metabolic activity stimulated by the development of anoxic conditions is denitrification, and an examination of the kinetics of induction of this activity can serve as a model of changes in other biologically catalyzed processes. The process of denitrification converts nitrogen oxides (nitrate and nitrite) to nitrous oxide and dinitrogen at a low level in most soils. Following soil pore-saturating rainfall, should all other requirements for denitrification be available, nitrogen

oxides are reduced to dinitrogen and/or nitrous oxide at rates several orders of magnitude greater than occurred prior to flooding of the soil. This stimulation of denitrification enzyme activity results from induction of denitrification enzymes under the oxygen-limited conditions imposed by water saturation of a soil. Smith and Tiedje (1979) found that upon oxygen depletion, two distinct phases of denitrification were observed: phase 1, in which nitrogen oxide reduction proceeded at rates supported by the quantities of previously existing denitrification enzymes, and phase 2, in which higher rates resulted from increased expression of genes encoding the key enzymes in the denitrification pathway. In their study, phase 2 began after 4–8 hours of anoxic conditions and was not dependent on significant microbial growth. Finally, should conditions conducive for denitrification persist, eventually the soil denitrification capacity would be enhanced by increases in population densities of the responsible bacteria. A variety of studies have shown field responses of denitrification activity reflective of these laboratory observations (Christensen et al. 1991a; Flühler et al. 1976; Focht 1974; Parsons et al. 1991; Sexton et al. 1985).

Changes in the chemical properties of soils can also result in the induction of previously unexpressed metabolic activities. For example, enzyme synthesis can be stimulated by various soil amendments – including naturally occurring inputs of plant or animal tissues or anthropogenic inputs such as fertilizers, sludge (i.e. sewage biosolids), compost or xenobiotic compounds, such as pesticides. As with denitrification, a lag in enzyme activity of a few hours following soil amendment is typically observed before a rapid increase in the enzyme activity is detected.

In summary, enzyme induction provides a mechanism for a rapid, short-term response of the microbial community to ecosystem perturbation. New enzymatic activity can be expressed in existing populations in as short a timeframe as 30 minutes to a few hours. After removal of the stimulus, enzymatic activities could return to preinduced levels as quickly as they were induced. The longevity of any soil enzymatic activity depends upon the stability of the proteins involved, the protein synthesis rate, and the rate at which the protein is decomposed by cellular or extracellular proteolytic activity. Therefore, enzyme induction could be viewed as a rapid biochemical response for maintenance of ecosystem homeostasis.

2.1.1.2 Cellular Replication and Soil Properties

Several characteristics of soil microbial communities must be considered when assessing the importance of cellular proliferation to the dynamics of metabolic function in any soil.

1) Soil microbial biomass is composed of a mixture of actively growing and dormant cells.
2) A wide range of population densities exist within soil microbial communities. Populations of individual species making up the microbial community vary

over several orders of magnitude – from as little as 100 or fewer individuals of some rare species per gram of soil to perhaps a million or more organisms of other species per gram.

3) The relative densities of various populations are determined by their ability to compete with neighboring organisms.
4) At least the minimal chemical, physical, and biological requirements of an individual microbial species must be met before growth and replication of that species can occur.
5) The chemical, physical, and biological properties of any soil site are constantly in a state of flux. As these properties change or are altered, the activities and nature of the microbial community are also modified.

In contrast to the relatively rapid response associated with induction of key enzymes discussed above, cellular proliferation is a slow adaptation to ecosystem modification but its impact on the ecosystem may be much more lasting. Enzymes or enzyme systems may be synthesized at rates that result in detectable levels of activity in a matter of hours. In contrast, changes in a metabolic rate occurring as a result of cellular proliferation may require several days or months to become detectable. The time limitations involved with cellular replication are shown when a bacterially catalyzed process is considered. Bacterial cells reproduce by binary fission with growth rates in soil of from less than one to about two or three cell divisions per day. Thus, since production of populations of several thousand cells may be necessary to yield a detectable change in metabolic activity, several days may be required for development of a sufficient cell mass to have a measurable impact on the soil community. Indeed, it may take several weeks of incubation in the presence of the growth-inducing substance before population densities of a few hundred individuals develop in situations where only a few cells with the requisite metabolic capacity existed prior to soil amendment. However, once the microbial populations have developed, they may survive as resting cells or spores for decades, or longer, even though the conditions causing their development no longer exist. Thus, a large population of inactive or resting cells may persist in a soil in which they are no longer capable of growth. Long-term survival is more commonly the exception (i.e. most nonfunctional, nonspore-forming biomass will be decomposed in periods of a few months to a year), but even this level of persistence of a phenotype in soil exceeds that resulting from enzyme induction and subsequent repression of the activity.

Relationship of enzyme induction and cellular proliferation to the concept of steady-state conditions: A discussion of replication of microbial cells and induction of nonexpressed activities requires a consideration of the concept of steady-state conditions in soil. For most scientists, an understanding of steadystate conditions is based on experience with chemical reactions. In the hypothetical reaction:

$$A + B \leftrightarrow C + D$$

an equilibrium state, or steady-state situation, is reached; that is, the forward and back reactions will reach equilibrium. For a given chemical reaction, the steady state is dependent upon the reaction constants for the forward and reverse reaction and the concentrations of the reactants and the products.

Within the reasonably defined situation of the test tube, predictable levels of all chemical species can be reached. Such is not the situation in a soil ecosystem. The concentrations of reactants, products, and levels of the enzymes involved are highly variable. Parameters affecting reaction conditions change with time, temperature, and certainly season. Therefore, if we wish to apply the steady-state concept to soils, we must develop a definition of steady-state that is applicable to soil biological populations.

One option would be to define steady state by the growth status of the microbial populations (i.e. population densities are unchanging). In axenic culture, this situation could be represented by stationary growth phase (see Chapter 4) but this condition is never really achieved in soil by actively respiring microbes. Growth of microbial cells is encouraged by consumption of microbial biomass by predator populations, augmentation of availability of carbon and energy supplies induced by diffusion of fixed carbon substances to the microbial cells, by mass transfer of nutrient laden water through the soil pores, or through root growth and death associated with plant community growth and decline, as well as by variation in soil physical properties, such as temperature and moisture. Furthermore, cell longevity may be reduced by the stress of coping with the harsh conditions of their environment. Thus, the microbial biomass could be envisioned as being in a constant state of flux.

Since both the microbial populations and their environment are steadily, albeit at times very slowly, changing, a chronological dimension to the definition of steady state must be applied. The fact that a variety of soil properties that control microbial population densities vary regularly on a daily and seasonal level suggests steady-state conditions may be determined by consideration of changes in mean population densities with time. For example, temperature cycles daily as well as seasonally. Nutrient levels available to microbial populations frequently relate to plant growing season since photosynthetically fixed carbon is the primary carbon and energy source for most soil microbes. To accommodate this regular cycling or fluctuation of soil properties controlling microbial activity, a steady state could be said to exist in a soil ecosystem when the cycles of microbial activity or proliferation are replicated on a regular (time) basis (e.g. seasonally, according to plant growth status, or annually).

Based on even this periodic variation of soil microbial properties and activity, a question could be raised regarding whether steady-state conditions really ever occur in soils. In reality, a true steady-state condition is rarely, or perhaps never, achieved in the highly variable soil environment, but many of our concepts and

models of soil systems are based on an assumption of steady state. Perhaps a more scientifically defensible expression of the status of the soil microbial community would be to modify our concept of steady state by designation of the occurrence of a *quasi* steady state rather than an absolute steady state condition for truly, a constant level of biological activity is never reached in any soil system, even on an annual basis. It would be rare to observe a field situation where exactly the same physical and chemical conditions impinging on microbial activity occur repeatedly year after year.

Similarly, considerations must be taken into account when evaluating the microbial activity at the field level at any single point in time. Again, as indicated in Chapter 1, the physical properties are extremely variable across a field surface, dependent upon such ecosystem-related properties as the distribution and nature of the plant community. Variation in microbial activity becomes even more acute as the level of the microsite is approached. This heterogeneity in levels of activity of a microbial process in soil has been clearly demonstrated (Christensen et al. 1991b; Parkin 1987; Parkin et al. 1987). For example, denitrification requires anoxic conditions plus metabolizable carbon sources and nitrate to occur. These necessities vary spatially and chronologically in soil. Parkin et al. (1987) found "hot spots" of high specific denitrification in soil, which were linked to microsites with elevated particulate organic carbon. In a soil core where a high denitrification rate was measured, 85% of the denitrification capacity of a 98 g sample was found in an 0.08 g subsample of the core. Thus, it is easy to postulate that depending upon the distribution of the microbial colonies and the substrates necessary for their function, activity could vary from essentially zero (no microbial cells present) to extremely high levels (large colonies in the vicinity of active plant roots). Again, the mean value of the activity in question over the field of study could be defined as the steady-state level of activity for the duration of the study. The "hot" or "cold" spots of activity may vary in intensity and location with time but the overall mean of metabolic activity in the field could remain reasonably constant.

In conclusion, a clear picture of the nature of steady state in a soil ecosystem could be likened to the daily conditions of any large city. When taken as a whole, the activity of the population appears to differ little on a daily basis (i.e. quasi steady state); cars and buses are streaming into the city and the sidewalks are clogged with people rushing to and from their offices. On any given weekday, the numbers of individuals associated with any given location appear to be reasonably constant. Yet, at the vantage point of a street corner, the situation appears to be nearly chaotic. Such is the activity of field soil as exemplified by the reactions occurring along a transect of any field site. Biological activities may be extremely variable along the transect (varying over several orders of magnitude), but an average value representative of the overall soil ecosystem along the transect can be calculated. Furthermore, the activity may be highly variable during the growing

season but again, a value representative of the process rates for the season may be estimated. In our model of the city, this variability could be likened to that involved with comparing weekdays with weekends. Operationally, the highly variable soil systems could be defined as being at a quasi steady-state level. An apparently chaotic system can be reduced to some semblance of reproducible order when integrated along a long transect or over the time frame of a growing or annual season. It is this potential for predictability and representation that makes possible the description of an ecosystem whose whole existence is based on processes occurring in highly variable microsites.

2.1.1.3 Cell Structure and Biochemical Stability in Soil

Amendment of soil with essentially any biodecomposable substrate, assuming conditions are appropriate for microbial metabolism to occur, results in a reasonably rapid depletion of the added material. In apparent contradiction to that observation is the fact that a wide variety of decomposable organic compounds contained in living cells are readily isolated from soil. That is, an apparent stability of labile substances is observed. These contrasting observations result from the definite cellular structure of microbial biomass. Readily decomposable organic compounds are protected within the cellular structure. Hence longevity of the biochemicals is not determined totally by the capacity of the microbial community to synthesize the enzymes necessary for their catabolism, but rather decomposition kinetics are determined, at least in part, by the capacity of the microbial biomass to breach the protective cellular barriers. In situations where the carbonaceous substrates are encased within protective cell walls, microbial populations may be starving in a soil containing large reserves of carbon and energy supplies that can support microbial cellular growth locked within protective cell walls.

2.1.1.4 Resting Structures and Soil Respiration

Another unique property of the soil biological system is that the cells may enter a metabolic state that allows them to survive under adverse conditions. Two levels of nonreplicating states of viable cells exist in soil. In the absence of appropriate energy supplies, microbes may enter a dormant or reduced metabolic state, or they may actually form resistant inactive structures (e.g. spores, endospores, sclerotia).

Whereas the impact of true resting structures, such as spores, on soil is minimal until they outgrow and form active vegetative cells, those cells in a resting (low activity) state have a continuous but low-level effect on the conditions of the microsite. These latter microbes must continue to consume energy to maintain their cellular structure. Thus, by oxidizing internal energy sources, they continue to consume free oxygen (respire) and producing carbon dioxide. At least a minimal

localized effect of the carbon dioxide generated from respiration associated with cellular maintenance would be expected to occur. Generation of this gas alters the atmosphere of the microsite where the resting microbial cell resides and increases the acidity of the interstitial soil water. For example, nitrifiers in the absence of their energy source, ammonium or nitrite, or any heterotroph existing in the absence of fixed carbon substrates may enter this situation where the only metabolic processes occurring in the cell are those associated with maintaining the cell structure. This form of existence could be pictured as a type of starvation where ultimate survival depends upon an eventual influx of an energy-supplying nutrient. Interestingly, microbes may exist for extended periods of time (decades to centuries for *Arthrobacter* sp.) under these conditions. For these microbes, cellular metabolic rates are reduced to those levels necessary to maintain a minimal cell structure (i.e. the level of enzyme synthesis machinery and metabolic tools necessary to synthesize the enzymes and cellular products necessary to take advantage of proper growth conditions when they develop). Considering the chronological as well as physical heterogeneity of organic carbon distribution within soil, it is logical to conclude that the majority of soil microbes are found either in this reduced metabolic state or in resting structures.

2.1.1.5 Microbial Activity and Soil Properties

Microbes are also unique in soil in that they can alter the solubility of soil mineral components, chemically reduce organic compounds to essentially undetectable levels, modify soil structure, oxidize inorganic compounds, and use a variety of soil components as electron acceptors. Merely through growth and metabolism, soil microbes alter their environment. For example, soil structure is enhanced by production of polysaccharides, which may link soil particles into macroaggregates (Chapter 1). In contrast, soil structure may be lost through oxidation of the colloidal organic matter supporting soil aggregate structure by the same microbes. A less obvious but nonetheless important aspect of the impact of microbial metabolism on soil particulates is the dissolution of soil minerals resulting from organic or inorganic acid production. A variety of organic acids, including acetic acid, citric acid, and lactic acid, carbonic acid (carbon dioxide) and mineral acids (sulfuric acid and nitric acid), are produced by the soil microbial biomass. These acids are direct contributors to the weathering of soil minerals.

Soil organic and inorganic components may also be oxidized or reduced as energy sources or electron acceptors for the microbial community. All nonphototrophic microorganisms must oxidize growth substrates for energy. Common reactions are organic carbon oxidation to carbon dioxide, oxidation of ammonium to nitrite and nitrite to nitrate, oxidation of ferrous to ferric iron, and elemental sulfur conversion to sulfuric acid (see Chapter 4).

The impact of these oxidative processes on soil is magnified by the nature of the final acceptor of the electrons produced by oxidation of the growth supporting substances. A basic principle of biochemistry is that all oxidative reactions must be balanced by comparable reductive processes. Thus, some environmental substances must be reduced as a result of microbial growth. Aerobic catabolism of organic carbon most commonly involves reduction of oxygen to water. Denitrifiers oxidize organic carbon compounds while reducing nitrate or nitrite to nitrous oxide and dinitrogen. Furthermore, carbon oxidation may result also in the reduction of sulfur oxides to sulfide or elemental sulfur, or the reduction of ferric to ferrous iron.

Practical implications of this basic biological principle become apparent when it is considered that both oxidative and reductive processes have major implications in reclamation of contaminated soils. Whereas aerobic, heterotrophic metabolism can be used to purify soils contaminated by organic compounds (e.g. hydrocarbons such as petroleum), reductive processes may be exploited for remediation of metal-contaminated and acid-impacted sites. For example, sulfate reduction to hydrogen sulfide may be facilitated in renovation of acid mine drainage-impacted sites (Mills 1985). Similarly, hydrogen sulfide production may be used to encourage removal of heavy metals from waters or immobilization of these metals in soil systems through precipitation as metal sulfides (see Wildung and Garland 1985). Frequently, a wetland-type system is developed to accomplish these tasks. Passage of metal-bearing water via overland flow through swampy ecosystems where hydrogen sulfide is generated results in precipitation of the metal sulfides.

2.1.1.6 Microbial Links to Aboveground Communities

It is relatively easy to forget that the processes occurring in soil cannot occur at maximum rates in the absence of fixed carbon inputs from the aboveground plant community. Some microbial activity is supported by catabolism of colloidal soil organic matter and more slowly decomposed biological components (complex polysaccharides, lignin, and lignin products), but maximal biomass productivity is derived from catabolism of more easily oxidized substrates, such as simple polysaccharides and proteins. Since the primary, natural source of such compounds in soil is plant biomass, a nearly essential dependency develops between the autotrophic aboveground community and soil microscopic life.

Similarly, the aboveground community is dependent upon the decomposer population in soil to mineralize macro- and micronutrients contained within dead plant biomass. For example, the organic nitrogen in the plant biomass must be converted to ammonium and nitrate before it can again be incorporated into new plant biomass. The facilitators of this cyclic process are the soil microbial community. (See Chapter 12 for further discussion of these processes.)

2.1.2 Implications of Microbial Properties for Handling of Soil Samples

An appreciation of the unique properties of living organisms in soil is mandatory for study and characterization of the versatile and rather fragile material called soil. A key point is that soil samples to be used to characterize native processes must not be treated in a manner destructive to the living systems contained therein. Of first consideration when collecting soil samples is remembering that the prime mover in the system is biological. Thus, the sample should be maintained so that biotic components are preserved and induction of major changes in their composition is avoided.

A primary example of a commonly used procedure in soil sample collection that necessarily results in alteration of soil biological activity is provided by an evaluation of the problems encountered by air drying soil samples. Historically, soil scientists have preserved soil samples by air drying. With this procedure, moist soil samples are incubated in the laboratory under conditions that allow soil moisture to approach ambient atmospheric levels. The final level of moisture retained in the soil may be considerably below that normally occurring under field conditions in that periodic influxes of water from rainfall or dew formation are precluded. This procedure is appropriate for preservation of many chemical properties of soil, but it is disastrous for evaluation of most soil microbial processes.

Air drying of soil creates a new ecosystem that may be quite different from that existing in the field from which the soil was collected. For soil microbes to function, there must be a coating of water on the soil particles (see Chapter 5). When soils are dried, sensitive microbes may die or be induced to enter into a resting stage. The fact that major changes in soil properties occur during soil desiccation is suggested by the observation that a burst of microbial respiration occurs when air-dried soils are remoistened. This increased respiration results from the oxidation of organic carbon liberated from soil aggregates disrupted by soil drying and from catabolism of the dead microbial biomass. This results in changes in the levels and nature of soil enzymatic activities, modification of soil organic components, and changes in the composition of the microbial community.

Thus, modification of the physical condition of soil can have a dramatic effect on the nature of the active microbial populations. This situation is particularly acute if these are the microbial communities of interest. For example, strictly anaerobic organisms are killed by contact with molecular oxygen. A gradient of sensitivity of such populations occurs, but in studies where quantification of such populations or their activities is desired (especially with anoxic soil samples such as swampy material), oxygenation of the soil must be avoided. Modification of the aeration status of soils from that existing *in situ* under oxygen-free or oxygen-limited conditions may also cause increased population densities of aerobic

microbes. In addition, a pool of partially decomposed fixed carbon substrates accumulates under oxygen-limited conditions. These substances provide carbon and energy for the aerobic microbes once free oxygen is introduced into the system, thereby resulting in augmentation of their population densities.

2.2 Measurement of Soil Microbial Biomass

Microbial biomass is a primary catalyst of biogeochemical processes in soil as well as an energy and nutrient reservoir. Its significance is exemplified not only by the infinite array of biochemical transformations catalyzed in soil but also by the quantities of fixed nitrogen it contains. Anderson and Domsch (1980) found that in 26 agricultural soils, nitrogen contained in the microbial biomass ranged form 0.5% to 15.3% of total soil nitrogen, with an average of approximately 5%. This nitrogen becomes available to the aboveground community upon death and decay of the microbial cells. In soils not receiving exogenously supplied fixed nitrogen (for example nonagriculture soils), this nitrogen pool is sufficiently large that its stability is a prime factor in controlling the flux of fixed nitrogen through the ecosystem. In disturbed soils, such as when grassland soils are initially cultivated, a decline in soil microbial biomass results in a release of large quantities of fixed nitrogen. The fixed nitrogen not incorporated into newly synthesized microbial cells or aboveground biomass is lost from the ecosystem through leaching to groundwater or through runoff. Hence, it is frequently useful to estimate the size of the soil microbial biomass and its stability.

Microbial biomass is readily assessed in simple ecosystems, such as the growth flask, but its measurement in soil is complicated by the complexity of the system and the fact that these cells comprise a small portion of the total soil mass. One could propose to estimate soil microbial biomass by counting the number of individual living units, e.g. cells for bacteria or mass of mycelium for fungi, in a soil sample using a microscope. These types of direct counts are frequently done, but it is essentially impossible to isolate all of the microbial cells from soil. In addition, direct microbial counts do not enable us to distinguish viable from dormant or nonviable cells, thus limiting the relevance of these counts. Alternative methods for estimating soil microbial biomass utilize various surrogates as indicators of the quantity of living cells present in a soil sample. Such a surrogate should be a reasonably easily quantified cellular component that can be extracted from soil. An ideal indicator of microbial biomass should be:

- present in all microbial cells
- found in all microbial cells at the same concentration, regardless of species designation

- present in the cells at the same concentration, independent of growth status
- rapidly decomposed upon death of the cell
- quantitatively extractable from soil
- easily assayed.

The last trait on this list, ease of analysis, is necessitated by the large number of samples that are generally processed and by the fact that the biomass may change during storage of the samples. A rapid, easily conducted procedure can allow avoidance of long storage times between sample collection and analysis. A variety of candidate substances for estimating soil microbial biomass have been used, but none meets all of the above criteria. Soil microbial biomass has been commonly estimated through:

- direct counting of microbes (Lundgren 1981; Paul and Johnson 1977; Rosser 1980; Sonderstrom 1977)
- analysis of specific cellular components, such as adenosine triphosphate (ATP), phospholipids, or muramic acid (Ausmus 1971; Fazio et al. 1979; Findlay et al. 1989; Jenkinson et al. 1979; King and White 1977; Paul and Johnson 1977; Verstraete et al. 1983)
- measurement of specific microbial processes, such as nitrogen mineralization (Alef et al. 1988) and reduction of dimethylsulfoxide to dimethylsulfide (Alef and Kleiner 1989)
- measurement of respiration rates (Anderson and Domsch 1973)
- direct analysis of cellular components solubilized by chloroform fumigation (Brookes et al. 1982; Sparling and West 1988; Tate et al. 1988; Vance et al. 1987a) or the carbon dioxide produced by respiration of these products (chloroform fumigation-incubation method) (Jenkinson 1976; Jenkinson and Powlson 1980).

Of these, the most commonly encountered procedures are direct counts, ATP analysis, respiration methods, and variations on the chloroform fumigation procedure. As noted below, these procedures are commonly used singly, but there are examples of studies where comparable results were achieved using multiple methods in parallel on the same samples (e.g. Fritze et al. 1996). It must be noted, however, that comparable results should not always be anticipated since the various procedures measure different parameters of the microbial biomass and therefore should not be anticipated to vary in concert. For example, the aerobic respiration methods commonly assess glucose metabolism whereas the chloroform fumigation-incubation approach relates to the quantities of cellular carbon released by the chloroform and subsequently mineralized by the surviving microbial community. We will discuss several of the most commonly used methods for estimating microbial biomass below, with an emphasis on the strengths and problems associated with each method. A more detailed presentation of these methods can be found in Parkinson and Paul (1982).

2.2.1 Direct Counting Methods

Soil bacterial and fungal biomass may be viewed directly by microscopic observation of preparations of soil samples. Typically, small quantities of soil are suspended and spread in a thin layer on a microscope slide or, alternatively, the liquid used to suspend the soil is extracted and the microbes contained therein collected on an appropriate filter medium. The slide or filter is dried and a portion of the preparation is examined under the microscope. One challenge with this approach is that the refraction index of microbial cells is not sufficiently different from that of some soil particulate organic components, making it difficult to differentiate microbial cells from nonliving soil components. A variety of methods have been developed to enhance microscopic resolution of microbial cells in soil. For example, fluoresecent DNA binding stains such as acridine orange (e.g. Mills and Bell 1986), diaminopimelic acid (DAPI) (e.g. Bottomley and Dughri 1989) and the SYBR dyes (e.g. Weinbauer et al. 1998) can be very effective for discriminating cells from other soil particles, and these stains have been widely used in estimating bacterial numbers in soils. Drawbacks of these stains include an inability to distinguish viable from dormant or nonviable cells and a lack of specificity, as these stains will bind to any double-stranded DNA they come into contact with.

Alternative approaches have been developed to overcome the shortcomings of these direct counting techniques. For example, metabolically active bacteria may be detected by treating soil samples with tetrazolium salts (water-insoluble formazan accumulates within metabolically active cells), labeling cells with radioactive substrates (autoradiography) (see Roszak and Colwell 1987) or by counting metabolically active bacteria. With the latter procedure, soil samples are incubated with naladixic acid, an inhibitor of DNA synthesis. The antibiotic amendment causes an elongation of metabolically active cells. The active cells can grow, but they cannot divide. These microbial cells may be detected in the treated soil preparations with acridine orange stain and epifluorescence microscopy. Direct microscopic counts can also be limited to specific microbial groups (species in some cases) through the use of fluorescently labeled antibodies (e.g. Demezas and Bottomley 1986) or fluorescently labeled oligonucleotide probes (e.g. Christensen et al. 1999).

Once the microbial cells have been quantified in several microscopic fields, the values are converted to biomass by multiplying the number of cells per unit of soil by their volume times the cell density. Calculation of the number of cells per unit of soil requires knowledge of the area of the microscope field and dilution of soil used in preparing the slide. Biovolume may be calculated from cellular dimensions estimated during their enumeration. Difficulties are encountered in converting the cell volume to cell mass (Bakken and Olsen 1983; Bratbak 1985; Bratbak and Dundas 1984; Van Veen and Paul 1979). Such conversion requires assumption of the impact of moisture on changes in cell dimension during sample preparation.

Once the counts of cells existent on the prepared slides are determined, along with the challenge of converting biovolume to biomass, problems arise regarding the validity of extrapolation of data collected from the limited area of the microscope slide to field-scale dimensions. These procedures generally use small quantities of soil (perhaps less than a gram), so some concern about extrapolation of the data to represent field or even ecosystem values is appropriate. Great care must be exercised with extrapolation of the direct count data since very small soil samples are generally used.

2.2.2 ATP Measure of Soil Microbial Biomass

For these estimates, soil is homogenized in a buffer solution to extract the ATP (Figure 2.1). Generally an acidic buffer is used to minimize solubilization of soil humic substances, which interfere with measurement of the ATP. The extractant is

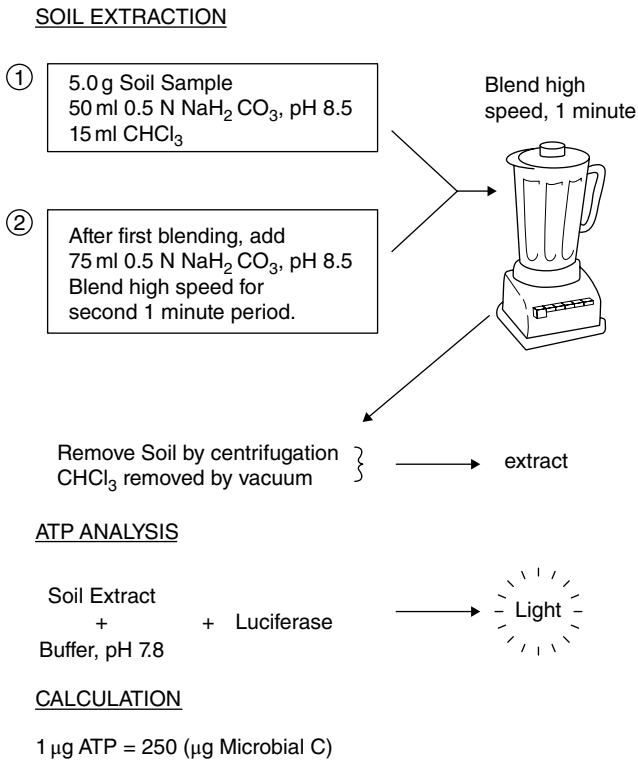


Figure 2.1 Outline of a typical ATP analysis procedure for estimating soil microbial biomass (see also Parkinson and Paul 1982).

removed from the soil suspension by centrifugation or filtration and the ATP is quantified by measuring light production with the firefly luciferin-luciferase system. Soil microbial biomass carbon is calculated by multiplying the measured ATP concentrations by a constant that is proportional to the amount of ATP contained per unit of microbial biomass as determined using a standard bacterial cell culture cultivated in laboratory media. The procedure is dependent upon the extraction efficiency of ATP from the soil sample and selection of an appropriate constant for final calculations.

An assumption underlying this procedure is that there is negligible variation in the ATP contents of microbial cells. The validity of this assumption is questionable since cellular ATP contents are highly dependent upon the metabolic status of the cell. Actively metabolizing microbial cells contain considerably more ATP than is found in resting cells. Recall that a major portion of the bacterial cells in surface soils exist in a resting or inactive stage. Furthermore, while the proportion of the cells that are active is unknown, it is reasonable to assume that this value varies between soils collected from different ecosystem types and within soils from the same ecosystem, depending on such soil properties as energy inputs and moisture levels. For further information on the use of ATP for microbial biomass measurements, see Ausmus (1971), Jenkinson et al. (1979), Ahmed and Oades (1984), Paul and Johnson (1977), and Verstraete et al. (1983).

2.2.3 Soil Aerobic Respiration Measurements

The use of soil respiration as an indicator of soil microbial biomass is based on the principle that the rate at which fixed carbon substrates are oxidized to carbon dioxide in a soil sample is proportional to the quantities of organisms mediating the reaction (Anderson and Domsch 1978) (Figure 2.2). With this procedure, soil samples must be collected and any plant roots or macroscopic biomass removed to negate carbon dioxide contributions from these sources. With the soil respiration procedure, an easily metabolizable carbon source, such as glucose, is added to the soil sample and carbon dioxide evolution measured with infrared analysis, gas chromatography, or should sufficiently large quantities of carbon dioxide be produced, by titration of the gas collected in alkaline solutions. Alternatively, ^{14}C-labeled substrates (e.g. ^{14}C-glucose) may be added to the soil sample and the ^{14}C-labeled carbon dioxide produced collected and quantified.

This procedure may be used to differentiate fungal and bacterial respiration in soil by amending the samples with population specific antibiotics (Anderson and Domsch 1973, 1975). Streptomycin is generally used to inhibit bacterial respiration and cycloheximide for eukaryotic respiration. Each antibiotic should be used singly and in combination since a proportion of the microbial population is resistant to both antibiotics. Furthermore, several concentrations of antibiotic must be tested with each soil studied to assure use of optimal antibiotic levels. Inhibition

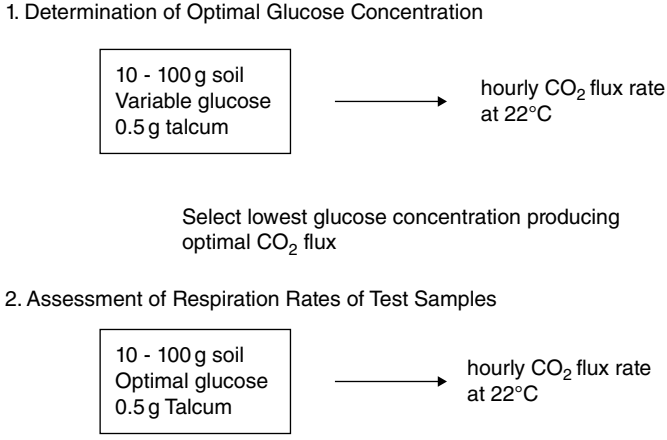


Figure 2.2 Outline of aerobic respiration method for estimating soil microbial biomass (see also Parkinson and Paul 1982).

of microbial activity is proportional to the quantity of antibiotic dissolved in the soil interstitial water. Antibiotics sorbed onto soil organic matter or clays are inactivated. Thus, the effective concentration of the antibiotic in soil may be significantly lower than the total quantity added. Higher antibiotic concentrations are required for inhibition of microbial populations in soils with high clay or colloidal organic matter contents than would be necessitated in a sandy soil, for example. Similarly, too much antibiotic must not be added to the soil since excessive antibiotic concentrations may inhibit the activity of nontarget microbes.

A major consideration in use of any technique involving measurement of carbon dioxide from soil is the soil pH (e.g. see Martens 1987). Microbial biomass may be underestimated by failure to recover all the carbon dioxide produced during the incubation period. In neutral or alkaline soils, carbon dioxide is retained in soil as bicarbonate or carbonate due to the following equilibrium:

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+ \rightarrow CO_3^{2-} + 2H^+$$

2.2.4 Chloroform Fumigation (Extraction and Incubation) Technique

Because of the general simplicity of this technique and the fact that it is relatively inexpensive, chloroform fumigation procedures are among the most commonly employed methods for estimation of soil microbial biomass. Thus, the

principles, applicability, and problems associated with the technique must be examined in detail.

Chloroform fumigation procedures (Figure 2.3) are based on (i) the disruption of cellular membranes by chloroform and (ii) quantification of some cellular constituent (e.g. carbon, nitrogen, phosphorus) contained in the biomass of the cells killed by the chloroform. Generally, chloroform vapors are used. Following fumigation, soils may be extracted directly and soluble carbon (e.g. Deluca 1998; Sparling and West 1988; Tate et al. 1988; Vance et al. 1987a), nitrogen compounds (e.g. Azam et al. 1988; Dahlin and Witter 1998; Gunapala and Scow 1998), or phosphate (Brookes et al. 1982, 1984; Hedley and Stewart 1982) measured (estimates of microbial biomass carbon, nitrogen, or phosphorus, respectively). Alternatively, for determination of microbial biomass carbon, the soils may be incubated to allow biological conversion of the dead microbial biomass to carbon dioxide to occur. This procedure is based on the assumption that the biomass carbon of the cells killed by the chloroform vapors is used by the surviving soil microbial biomass as a carbon and energy source. (Note that aerobic incubation conditions are required.

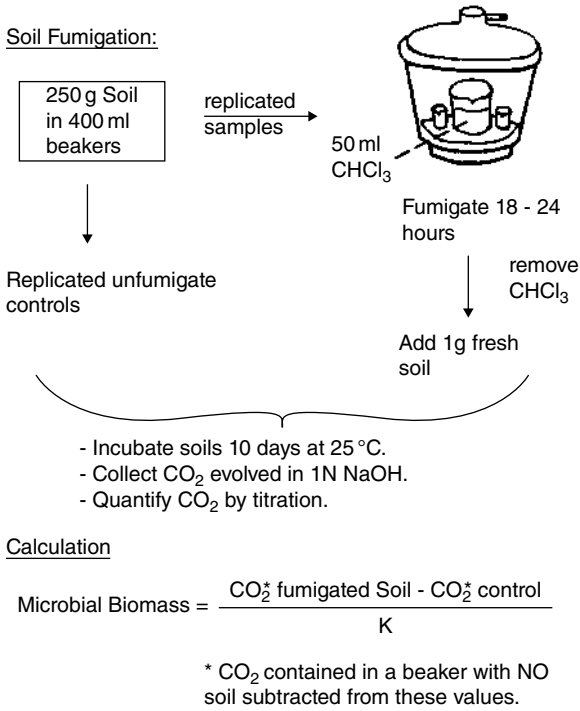


Figure 2.3 Outline of the chloroform fumigation-incubation procedure for estimation of soil microbial biomass (see also Parkinson and Paul 1982).

Anaerobic conditions result in incomplete oxidation of the cellular carbon of the dead cells.) To assure existence of adequate microbial populations during the incubation period, the fumigated and control soil samples are usually inoculated by amendment of the chloroform-treated soil with small quantities of nonfumigated soil (Chapman 1987). For assessment of carbon dioxide production, soils are sealed in respiration chambers. Carbon dioxide is either collected in alkaline solutions and titrated or assayed with gas chromatographic procedures.

For each of the procedures – incubation or direct extraction methods – assay of nonfumigated soil serves as a control. The total biomass present is calculated by subtracting quantities of carbon dioxide produced in nonfumigated soil samples (controls) from that yielded in the fumigated soil samples and dividing by a constant. The constant is a value representative of the killing efficiency of the fumigation procedure. Most frequently, for assessment of microbial biomass carbon, killing efficiency is considered to be about 40% (i.e. a constant of 0.4). Different values for the constant are used when quantifying microbial biomass nitrogen (Azam et al. 1988) and subsurface soils (e.g. Dictor et al. 1998; Tessier et al. 1998). Since the killing efficacy of the chloroform varies with such soil properties as moisture and type, it is imperative that the formula used to convert the raw data to biomass carbon be stated so that the data may be recalculated should it be necessary to compare them with values from other studies where alternative calculation procedures were used.

Some variation in incubation period of the soil samples following chloroform fumigation has been used. The treatment and control samples may be incubated concurrently for 10 days and total carbon dioxide yield assayed, or the control sample could be incubated for 20 days with carbon dioxide evolved between days 10 and 20 being used in the calculations. In this situation, for the calculations, the carbon dioxide produced in control samples during the last 10 days of a 20-day incubation period is subtracted from that yielded in the fumigated samples during the first 10 days of incubation. This procedure avoids inclusion of the commonly observed burst of microbial activity in disturbed soils samples (as would be anticipated to occur with control soils) in the final calculation. Failure to compensate for this soil disturbance-induced carbon dioxide production may result in an underestimation of total soil microbial biomass.

Aside from providing a reasonable estimation of the quantities of cellular components (carbon, nitrogen, and phosphorus) contained in microbial biomass, chloroform fumigation of soils is a valuable tool for demonstration of assimilation of soil amendments by the soil microbial community. For example, Kassim et al. (1982) quantified the incorporation of glucose, acetate, pyruvate, uracil, uridine, amino acids and a variety of polysaccharides into microbial biomass. To accomplish this, ^{14}C-labeled substrates were added to soil samples and then the amended soil samples were incubated for varying time periods to allow their incorporation into various soil organic matter fractions, including microbial biomass. Following

incubation, the nonreacted ^{14}C-labeled amendment was washed from the soil. For analysis of the microbial biomass-incorporated materials, the soil samples were fumigated with chloroform. Assessment of the excess ^{14}C-labeled carbon dioxide produced during the incubation period in the fumigated samples provided an estimation of the quantity of the metabolized carbon incorporated into microbial cellular components.

The chloroform fumigation procedure is an effective means for estimating microbial biomass in most soils, but difficulties have been encountered with application of the technique to acidic soils (Coûteaux et al. 1989; Vance et al. 1987a, 1987b), wet soils (Ross 1987, 1988) and in plant biomass-amended soils (Martens 1985). A common trait among these situations is that the capacity of the microbial community to recover following fumigation and subsequent catabolism of the dead microbial cell material has been affected. Large quantities of available carbon preexisting in the soil samples may result in minimal differences between quantities of carbon dioxide produced by control and fumigated soils (i.e. the carbon dioxide production from the killed biomass oxidation is not significantly greater than the large quantities produced from catabolism of native soil organic matter). In extremely acidic soils, data suggest that the nature of the population (fungal vs bacterial contributions to carbon metabolism) may differ between fumigated and control soils (Tate 1991). In either case, modification of the incubation procedure and/or method of calculation of the microbial biomass may be necessary (Vance et al. 1987c).

2.2.5 Limitations of Microbial Biomass Measurements

A variety of inaccuracies are intrinsically associated with estimation of soil microbial biomass. (Note that in every case, the word "estimate" not "measure" is used in conjunction with the value produced from the microbial biomass assessments.) In selecting procedures, determining constants for calculation of results and interpreting the data, basic properties of the soil must be considered. These include the soil moisture level, pH, and organic matter contents referred to above as well as soil texture. Extraction efficiency of charged materials may be reduced in soils with high clay contents. Furthermore, high levels of colloidal soil organic matter may sorb products of interest or interfere with analysis (ATP-luciferin-luciferase assay).

It should also be noted that the calculation for each of the procedures described above requires use of a constant. Selection of the control value assumes a specific effect of soil drying on biovolume for direct counting procedures, a concentration of ATP in cells of soil microbes for the ATP-based assay, or a killing efficiency for chloroform fumigation. All such assumptions have a leveling effect on the data for they may be true for some samples or cells and not for others.

The above difficulties must be considered by the investigator in data analysis but are to a certain degree beyond experimental control. Other procedures that are within design control involve laboratory and field procedural variables. Nearly all procedures require some degree of sample storage prior to analysis. Once soil samples are collected, creation of a new ecosystem commences. Soil properties limiting microbial activity in the field site are removed (e.g. perhaps temperature, soil structural occlusion of organic matter, moisture variation) and new delimiters of microbial population densities and activities are created. Such changes must be minimized. The most reasonable means of reducing artifacts due to sample preparation procedures is to assay the microbial biomass as quickly after collection as possible (preferably within minutes to a few hours). Should this not be possible, storage at 4 °C and avoidance of direct sunlight are mandatory. Some studies suggest that soil samples can be stored at −20 °C for extended periods without major changes in some measures of microbial biomass and activity (e.g. Stenberg et al. 1998). It is imperative that control studies be conducted to validate storage procedures since utility of a particular storage method may vary with soil or ecosystem source as well as with assay method. Good laboratory practice requires documentation of all sample preparation procedures so that any compromising of the results can be determined.

Finally, the researcher must ask the question, "What is being measured by the procedure used?" Some methods provide an estimation of the total microbial populations present (e.g. chloroform fumigation procedures or ATP analysis), whereas other methods only quantify the microbes capable of a particular reaction. For example, utilization of glucose in soil aerobic respiration measurements provides a good estimate of the biomass of organisms capable of oxidizing glucose as an energy source. In the former situation (total biomass quantification), both active and inactive microbial populations are quantified whereas in the latter (soil aerobic respiration assessment), only those capable of catabolizing a particular biochemical process are enumerated.

2.3 The Nature of Soil Inhabitants

All seven major groups of microorganisms plus higher animals interact in soil to catalyze the biogeochemical processes occurring therein. Soil bacteria, archaea, fungi, actinomycetes, protozoa, algae, and viruses, as well as nematodes and mite populations, contribute to development of the total community. The arrangement of these classes of organisms may be evaluated in a hierarchical manner. This association provides the basis for fundamental viewpoints in examining native ecosystems. The bottom of this arrangement is the *individual* organism. The summation of the individuals within a given taxonomic or functional group is a *population*.

The totality of all populations of different organisms in soil constitutes the *community*, and the interaction of all biological components with the abiotic portions of the environment constitutes the *ecosystem*.

Soil ecological research can be divided by the portion of this hierarchy stressed in the study. Such research may have an autecological or synecological basis. An examination of the behavior of a single species in an ecosystem, including a study of the effect of chemical, biological, and physical aspects of the ecosystem on the population is an *autecological study*. In contrast, soil may be examined as a whole. In this situation, soil is being treated as if it were a single "tissue." Thus, the relationships between the environment and community of organisms (*synecology*) is being stressed. For synecological research, processes are of more concern than the identity of the individual microbes involved. For example, denitrification kinetics and its variation between soil types may be studied (synecology) or a researcher may concentrate on the identity of the individual denitrifying species existent in a soil site (autecology).

2.4 Autecology and Soil Microbiology

Quantification of microbial biomass is useful for expressing ecosystem potential (e.g. nutrient or energy cycling, or even general biological capacity), but most evaluations of soil community activity require elucidation of specific biological or biochemical processes and their kinetics. The processes themselves may be the center of the experiments (synecology) (see Section 2.5) or elucidation of the specific microbes and the system properties controlling their activity may be the pivotal point of the research (autecology). The latter aspect of ecosystem evaluation is the topic of this section.

The starting point for discussion of autecological research is a determination of what constitutes a bacterial species. An autecological study by strict definition involves assessment of the presence or activity of individual species. This is reasonably readily accomplished with higher organisms where species designations are more easily assayed, but the fluidity of bacterial species complicates such research for the soil microbiologist. This difficulty arises from the reasonably easy exchange of genetic material that occurs between bacteria via horizontal (or lateral) gene transfer. The definition of a bacterial species resides in the genetic material contained therein and its expression as it impacts cellular morphology, colonial structure and metabolic capacity. Should the genetic material resulting in distinctive properties that define one of the bacterial species be readily transferred into another species, the distinction between the two groups of bacteria becomes questionable at best. Such problems with speciation of soil bacteria are reasonably common.

Questionable or difficult separation of bacterial isolates into species has led to other more easily derived groupings of bacteria. Thus, autecological research may

involve the study of bacterial groupings separated by phenotypic traits (functional or even colonial groupings, i.e. guilds) rather than by species designation (Mills and Bell 1986). Alternatively, an autecological study, in principle, may involve analysis of DNA sequences with a selected percent similarity as the basis for species designation (see Chapter 3.). Thus, the discussion of autecological research that follows is based on the expanded definition of autecology, which allows for the study of guilds as well as scientifically accepted species designations.

2.4.1 Limitations to Autecological Research

It must be noted that the compromise involved in delineation of bacterial species incorporates a limitation into the value of the data derived from such studies. A primary objective of any ecological study is not only to understand the particular system of study, but to develop principles applicable to a variety of situations. Taxonomic designation of the living organisms studied has provided a common link between research projects. The phrase, "a lion is a lion no matter where it is encountered" can be readily accepted. To some degree, such a statement could be made for many of the historically studied bacterial species, but a generalization of this type regarding bacteria is questionable. Thus, use of the less restrictively derived guilds limits intersystem and interlaboratory comparisons.

A second difficulty associated with autecological research results from the minute dimensions of soil microbes. Autecological research provides meaningful data for understanding biological processes, but extrapolation of such research to an ecosystem scale is limited. Data are collected clearly describing the interactions of microbial colonies and their environment, but the area impacted by an individual microbial colony in soil encompasses a few cubic microns at best. The inherently small size of the microsite impacted by the individual microbe necessitates collection of large quantities of data before a clear picture of the effect of the totality of soil microbial colonies emerges (Tate 1986). Development of automated microbiological procedures for species identification, expanded use of fluorescent antibodies (including monoclonal antibodies), and application of DNA-based methods to detection of individual microbial species and quantification of their density in soils should result in an expansion of autecological soil microbiological research.

2.4.2 Autecological Methods

2.4.2.1 Viable Counts/Enrichment Cultures

Prior to the development of methods for detecting nucleic acid sequences specific for selected microbes and the application of these methods to physically and chemically complex systems like soil, the initial step in any autecological study of soil involved growth of the microbes of interest in axenic culture. Indeed, this

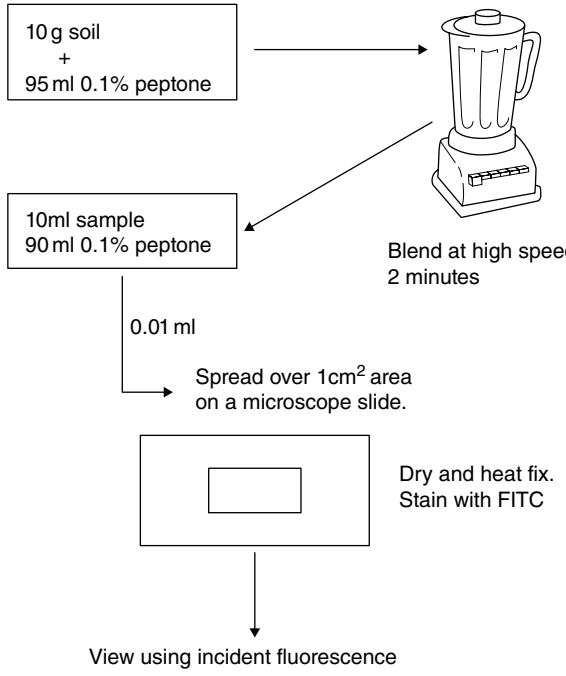
remains the initiation point – and in many cases the primary limitation – for most such research today. Exceptions to this observation are exemplified by studies in which the experimenter's interest resides simply with detecting selected microbes and estimating their population density. In that situation, indicators such as species-specific activities or DNA-based techniques eliminate the necessity of some cultural studies.

Microbes may be isolated from soil using nonselective techniques, such as growth on soil extract agar (perhaps this could better be termed "less selective" since growth in any laboratory medium allows growth of only a portion of the microbial population), or selective techniques, such as selective growth and enrichment culture. With the latter two procedures, microbes with certain desired physiological properties are directly sought. With enrichment cultures, increases in microbes existing in low population densities are favored prior to isolation of the axenic cultures, whereas with selective growth an extract of soil may be cultured directly on selective media.

The first concern in enumerating specific soil microbes, especially bacteria, results from their population density. Typically bacteria exist in soil at densities of 10^8–10^9 colony forming units per gram dry soil. These are associated with 10^7–10^9 actinomycete propagules per gram dry soil and about 10^6 fungal and protozoan propagules each per gram dry soil. Direct plating on a nonselective agar medium would clearly be unfruitful. Soil samples must therefore be diluted to reduce colonial growth on agar plates to 20–300 colonies per plate (Figure 2.4). Consideration of the dilution factor plus the quantity of diluent added to the growth medium allows calculation of viable microbes in the original soil sample. A result of this necessity to dilute soil samples is that the data from such studies are highly variable. A slight variation in dilution precision – especially at the lower dilutions – causes large differences in estimated propagule densities. Variations as large as plus or minus 100% are not uncommon.

With viable counts of microbes from any environmental sample, a qualification must be made regarding the meaning of "viable." Those bacteria classified as viable for any specific experiment in reality only include the propagules capable of growth on the media used and only under the particular incubation conditions employed. Comparison of data from direct microscopic analysis of soil samples with results from viable plate counts reveals that in most cases only about 0.1–10% of the observed bacteria grow in culture, a phenomenon known as the "great plate count anomaly" (Staley and Konopka 1985).

A further complication associated with microbiological analyses of individual species in soil samples results from the variation in microbial growth rates. Typically, those organisms that form visible colonies during the most commonly used incubation times are those with the shortest generation times. Thus, many organisms that are important *in situ* are overgrown. This difficulty may be



Calculation:

Biomass ∞ Number of bacterial soil × Bacterial volume × Specific gravity × Carbon content

Figure 2.4 Outline of procedure for estimation of microbial biomass using direct observation of fluorescein isothiocyanate (FITC) label. See Schmidt and Paul (1982) for details of procedure.

overcome by using selective media (for example, media amended with antibiotics to which the desired population is resistant but most soil bacteria or fungi are sensitive or media containing selective growth substrates) or enrichment cultures, again based on some unique property of the microbe(s) of interest.

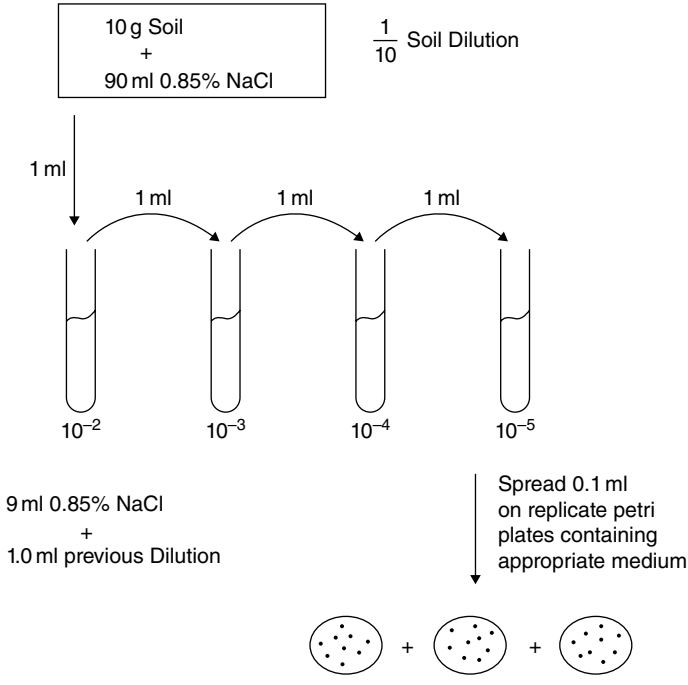
Viable plate counts are also used for enumeration of soil fungal populations. Estimates of fungal population densities in soil is confounded by their prolific spore production. Immense quantities of spores may exist in soil with few or none of the organisms existent as hyphae. Generally, fungal population densities are best estimated by direct microscopic observation.

2.4.2.2 Most Probable Number Procedures

A variation of the viable count procedure is the most probable number estimation of microbial populations. Many microbial populations (e.g. a variety of protozoa,

nitrifiers, and sulfur-reducing bacteria) are difficult to grow in defined media. Also, when all potential contributors to a given metabolic function, perhaps nitrification, are to be quantified, it must be realized that some individual species contributing to the activity may have been grown in axenic culture, but the probability frequently exists that other nonculturable strains occur in soil. Most probable number procedures may provide a measure of the density of such populations (Figure 2.5).

Most probable number procedures are based on determination of the dilution of the soil sample beyond which no propagule of the population to be quantified can be detected, i.e. the extinction point. Therefore, with this method soil must be diluted until no further propagules of the organisms of interest are present in the highest dilution prepared and multiple tubes of culture media (e.g. five per dilution) are inoculated from each dilution. After an appropriate incubation period, a trait common to all of the organisms to be quantified is measured (visual observation of



Calculation:

$$\text{Viable cells/g dry soil} = \frac{\text{Mean Plate Count} \times \text{Dilution Factor}}{\text{Dry weight of soil}}$$

Figure 2.5 Outline of viable plate count method for estimating numbers of organisms per gram of soil.

protozoan cells, nitrate or nitrite production for nitrifiers, or black color produced by precipitation of iron sulfide, for the examples listed above). Population densities are determined using prepared tables relating to the statistical probability of the presence of the organisms of interest to the number of positive samples for each dilution (e.g. Alexander 1982). This procedure retains the problems of selectivity of media and incubation conditions. Furthermore, due to the nature of the calculation procedure and the range of dilutions used, confidence limits are frequently as great as plus and minus 300%.

2.4.2.3 Sources of Error in Viable Count Procedures

Each step in any viable count procedure (soil sample collection and storage, preparation of dilutions, colony development) contains major impediments for production of an accurate population census. The most fundamental of these is the necessity of collecting site representative soil samples. Once attained, the soil must be stored in a manner that precludes major changes in the population density. Fortunately, considering the intrinsic variation of the dilution procedure itself, some change in the microbial populations can occur before statistically significant effects are detected. That is, with reasonable sample storage procedures, changes in microbial population densities are usually less than the inherent variation of plate count or most probable number data. For most studies, storage of the soil samples for limited times at 4 °C results in minimal changes in the data.

Two sources of data variation incurred during preparation of soil dilutions may be controlled by careful attention to proper laboratory procedures and experimental protocol (these are variability due to dilution procedure precision and the potential for microbial growth during dilution preparation). A third source of error is basic to the procedure – association of microbial cells with soil particles – and likely must be considered during data interpretation, but little can be done to prevent the problem. Association of microbial propagules with soil particulates may result in an underestimation of their number. Typically, microbial colonies in soil consist of a few cells adsorbed to a soil particle or linked through production of a polysaccharide slime. Incomplete mixing of the soil in the diluent could result in data that are an estimate of soil particle-bound colonies rather than separated microbial propagules. This data compromise can be minimized by thoroughly suspending and mixing the soil sample in the diluent, but realistically it cannot be eliminated.

A problem frequently encountered even when extreme caution is used in preparing the dilutions is microbial growth in the dilution media. Microorganisms generally have generation times of several hours in native soil samples. Thus, it might be considered that time lapse from preparation of the initial dilution to plating of the sample has little effect on the colonies developing on the plate. Unfortunately, this assumption is not true. Mixing of the soil sample in the dilution medium liberates metabolizable organic material from the soil. The solubolized organic carbon may

precipitate a few rapid divisions of the soil heterotrophic microbes under the less restrictive conditions of the dilution tube. This problem is particularly acute when the diluent contains mineralizable organic carbon. For example, it is not unusual to prepare dilutions in 0.1% (w/v) amino acids or 0.1 strength of a growth medium such as trypticase soy broth to minimize the impact of dilution on fragile microbial cells. Hence, it is desirable to minimize the time lapse between preparation of the initial dilution and inoculation of the growth medium.

2.4.2.4 Interpretation of Viable Count Data

Interpretation of viable count data must be based on the knowledge that (i) not all soil microbes are capable of growth on the medium and (ii) those organism detected are a mixture of organisms that were active and those which were inactive *in situ*. As indicated above, the number of colonies developing on the growth media represents only the microbes capable of growth on the selected medium under the prevailing incubation conditions. This is particularly true when selective media are used. These media are necessarily more stringent than those selected for estimation of total microbial populations (e.g. bacterial or fungal population densities). For example, it is not unusual to attempt to isolate organisms capable of using a particular pesticide as a carbon and energy source. To do this, a medium is prepared containing nitrogen, phosphorus, sulfur, and trace minerals necessary for growth and buffered at an appropriate pH for population development. A concentration of pesticide is added to the salts medium that is sufficient for colonial development, but not toxic. This medium appears to be ideal for the study, yet no microbes grow or only a few species are isolated from a soil sample in which it is known that a diverse population capable of catabolizing the pesticide exists. A limiting factor associated with the medium is that all microbes that require vitamins or amino acids are precluded from growth (see Figure 2.6).

A further caveat regarding interpretation of viable count data relates to the nature of the state of the microbial populations in soil. Production of a colony on the test medium indicates that the particular organism was viable in the soil of interest, but nothing is indicated regarding the activity of the microbe in the native soil sample. Actively growing cells, nongrowing cells, and resting cells will develop colonies under appropriate conditions. Thus, isolation of a particular organism in axenic culture solely means that the organism was present in the soil sample, not that it contributed to the metabolic activity expressed in the soil at the time of sampling.

2.4.3 PCR for Quantification of Soil Microbes (see Figure 2.7)

Soil microorganisms can also be quantified using a modification of the standard polymerase chain reaction (PCR) known as real-time PCR. Standard PCR makes copies of a targeted DNA sequence through repeated cycles of *in vitro* DNA synthesis.

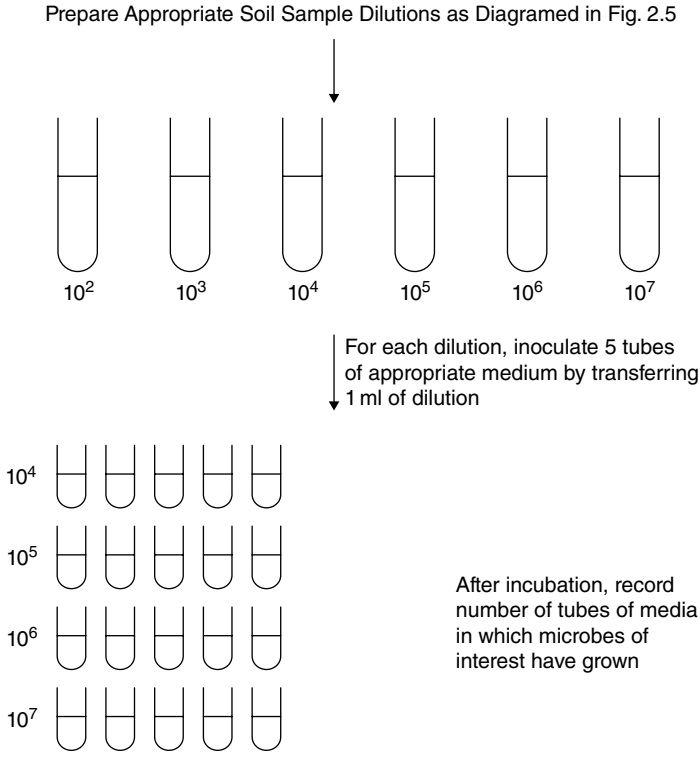


Figure 2.6 Outline of most probable number method for estimating number of organisms per gram of soil.

PCR depends on some knowledge of the DNA sequence to be copied so that primers complementary to regions flanking the targeted DNA sequence can be designed and employed in the assay. PCR also depends on the use of a heat-stable DNA polymerase, e.g. DNA polymerase isolated or cloned from *Thermus aquaticus*, a thermophilic bacterium, because thermal cycling is used to achieve repeated rounds of DNA denaturing, primer annealing and DNA synthesis. PCR results in exponential amplification of the target DNA sequence, resulting in thousands to millions of copies of the sequence. The application of PCR-based assays to soil microbiology has revolutionized our understanding of soil microbial communities and has enabled a variety of approaches for the exploration of soil microbial diversity. Chapter 3 will discuss these approaches in detail.

A modification of the standard PCR assay can be used for quantification of soil microorganisms. This modification, known as real-time PCR, enables the progress of the PCR reaction to be assessed after each round of amplification. As explained below, this real-time monitoring enables the user to determine the copy number of the target DNA sequence in an unknown sample, thus making the

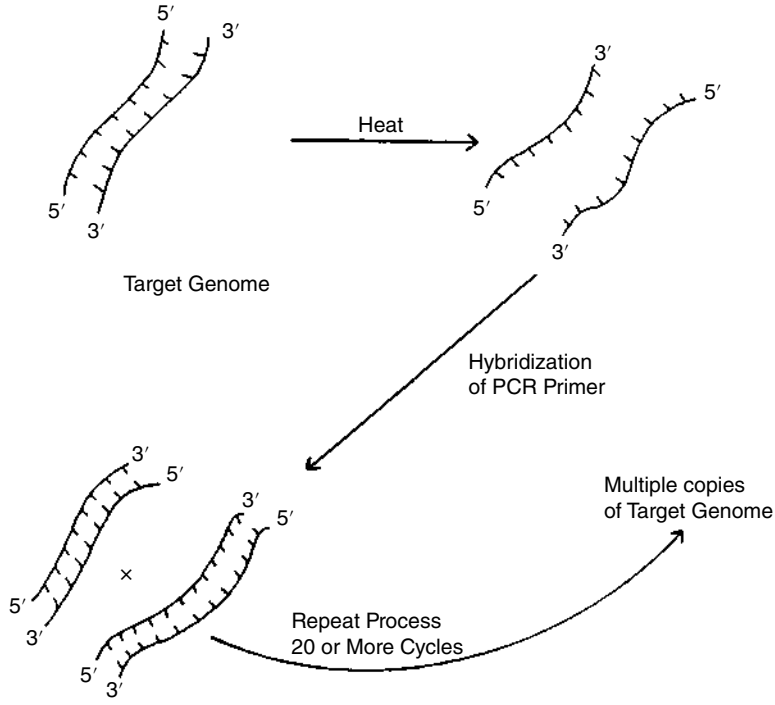


Figure 2.7 Outline of polymerase chain reaction (PCR) process for amplifying specific DNA sequences.

assay quantitative, leading to the term quantitative PCR (qPCR). There are two methods that can be used to accomplish real-time qPCR, SYBR-based assays and Taq-man assays. Both of these approaches depend on the monitoring of fluorescent reporters as indicators of the progress of the reaction, and so both require a specialized thermal cycler with the capability of monitoring fluorescence in each reaction tube after each round of replication.

2.4.3.1 SYBR Method for Real-Time PCR

SYBR dyes bind to double-stranded DNA and fluoresce only when bound. In a SYBR-based real-time PCR assay, a SYBR dye such as SYBR Green is included in the PCR mixture and fluorescence in each of the PCR reaction tubes (or wells if run in a microwell plate) is measured after each round of replication using a specialized thermal cycler (as mentioned above). As the reaction proceeds and more copies of the target DNA are produced, this will increase the amount of double-stranded DNA in the reaction tube, which will increase the fluorescent signal from that reaction due to more binding of SYBR Green molecules. For a given PCR

reaction, plotting the fluorescent signal against cycle number will produce an S-shaped curve, with an exponential increase followed by a plateau. It is possible to define a threshold level of fluorescence, generally near the inflection point at which fluorescence begins to increase most rapidly, such that the number of cycles required to cross this threshold (the C_T value) can be correlated with the number of copies of the target DNA sequence present in the PCR reaction at the start of the assay (i.e. cycle 0). To quantify the target DNA in a sample with an unknown concentration of the target (e.g. DNA extracted from a soil sample), a series of DNA standards of known copy number are run in parallel with the unknown sample. These standards are generally a dilution series of genomic DNA from a pure culture of an organism known to contain the target DNA sequence, but in some cases clones of the target DNA sequence can also be used as standards. After running the qPCR reaction, the standards are used to generate a standard curve of copy number vs C_T value. This standard curve can be used to determine the copy numbers of the target in the unknown samples based on their measured CT values.

One significant limitation of SYBR-based qPCR is the lack of specificity of the SYBR dyes. These dyes will bind to any double-stranded DNA, so any nonspecific amplification or primer-dimer formation that occurs during the PCR reaction will contribute to the measured fluorescent signal. It is thus critically important that primers and cycling parameters (especially the annealing temperature) be chosen carefully and validated experimentally. To validate reaction specificity, most SYBR-based qPCR reactions include the determination of a melting profile at the conclusion of the amplification cycles to verify the production of only one product. Most qPCR thermal cyclers can be programmed to determine a melting profile immediately at the conclusion of the PCR cycles. Melting profiles are produced by running the products of the PCR reactions through a range of temperatures, for example from 50 to 95 °C, and reading the fluorescent signal at set increments, for example after every 1 °C increase. This increase in temperature will cause the PCR amplicons to denature, resulting in a decrease in double-stranded DNA and thus a decrease in the fluorescence produced by bound SYBR dye molecules. For a single PCR product, the melting curve should show a rapid decrease with a steep slope at the melting temperature of that specific DNA fragment.

The melting temperature of a given DNA fragment depends on the length and the guanine-cytosine (GC) content, and to a lesser degree on the specific base sequence, so if a PCR reaction produces more than one product (due to nonspecific amplification or the formation of primer-dimers) these products will have different melting temperatures and the melting curve will show more than one inflection point. The melting profile is thus an extremely useful way to verify the production of only one product in a qPCR reaction. Additionally, the products of the qPCR reactions are usually analyzed by gel electrophoresis to verify the production of a single product of the expected size.

2.4.3.2 Taq-Man Method for Real-Time PCR

The Taq-Man approach to qPCR is similar to the SYBR approach, but differs in the fluorescent reporter used. The TaqMan approach includes a probe, which is a short DNA fragment (~20 bases) that is complementary to a region within the target DNA sequence. This probe is synthesized with a fluorophore attached to the 5′ end and a quencher at the 3′ end. The quencher blocks the fluorescence of the fluorophore when both are in close physical proximity, e.g. if both are attached to the probe. In the Taq-Man approach many copies of this labeled probe are included in the qPCR reaction and during each annealing step the primers, which flank the target DNA sequence, and the probe molecules, which target a region within the target sequence, will bind to their complementary regions. When DNA polymerase copies the target sequence by extending one of the primers it eventually runs into the probe that is hybridized to the template strand, and the 5′ to 3′ exonuclease activity of the polymerase degrades the probe so that it can continue its DNA synthesis. Degradation of the probe releases the fluorophore from the 5′ end of the probe, freeing the fluorophore from close proximity to the quencher, resulting in fluorescence of the fluorophore. Therefore, in the Taq-Man qPCR assay, the fluorescent signal is proportional to the number of copies of the target gene. As with the SYBR-based qPCR assay discussed above, the copy number of a target DNA sequence in an unknown sample can be determined by generating a standard curve of fluorescence vs cycle number using a series of DNA standards of known copy number. This standard curve can be then used to determine the copy numbers of the target DNA sequence within the unknown samples based on their measured C_T values.

The Taq-Man assay has a much higher specificity than the SYBR-based assay because with the Taq-Man approach fluorescence is only produced when the probe binds to the specific target DNA sequence and is subsequently degraded. Due to the nature of the fluorescent reporter system, the Taq-Man approach does not enable the determination of melting profiles, but the higher specificity of the Taq-Man assay reduces the need for this analysis. The specificity of the Taq-Man approach is a significant advantage over to the SYBR-based approach. However, the design of the internal probe for the Taq-Man approach does require more knowledge of the target sequence and it requires that a region of sequence conservation exist within the target DNA sequence. It can be difficult to meet these requirements for some qPCR applications and for some target DNAs, making the SYBR approach a more appealing option in some cases.

2.4.3.3 Applications of Quantitative Real-Time PCR to Soil Microbiology

Quantitative PCR can be used to quantify any target DNA sequence as long as suitable primers can be designed, although qPCR does work best for relatively short amplicons (500 bases or less). For quantification of soil bacteria primers targeting

the 16S rRNA gene can be used (e.g. Fierer et al. 2005; Okano et al. 2004) as this gene is present in all bacteria and it contains regions that are sufficiently well conserved to allow for the design of primers that will target all bacterial species, so called universal primers. It is also possible to use qPCR to target more specific taxonomic groups of bacteria by designing probes to target 16S rRNA gene sequences that are specific for the taxonomic group of interest. It is also possible to use qPCR to target copy numbers of genes encoding enzymes involved in specific metabolic pathways, also known as functional genes. The use of functional gene primers allows quantification of microorganisms involved in specific soil processes. For example, ammonia-oxidizing bacteria and archaea have been quantified in soil using primers targeting their respective *amoA* genes, which encode the alpha subunit of the ammonia monoxygenase enzyme (Leininger et al. 2006). Denitrifying bacteria have also been quantified in soil using primers targeting several genes involved in the denitrification pathway, including *nirS*, *nirK* and *nosZ* (Wallenstein and Vilgalys 2005). Finally, qPCR approaches have been used to quantify fungi in soils based on primers targeting eukaryotic rRNA genes (Bates and Garcia-Pichel 2009) or the rRNA intergenic transcribed spacer (ITS) region (Fierer et al. 2005).

2.4.3.4 Limitations of qPCR Approaches

Quantitative PCR is a powerful tool for the quantification of a wide array of gene targets in soil, but it has significant limitations that must be considered when assessing data produced with this approach. For example, qPCR can be used to determine copy numbers of target DNA sequences within soil samples, and gene copy numbers can be used as a proxy for the population size of the organisms containing the target gene. However, it is not possible to convert gene copy numbers to cell numbers because there is not a consistent relationship between copy numbers of various genes and cell numbers. For example, bacterial genomes have been found to have as few as one and as many as 13 copies of the rRNA operon (Klappenbach et al. 2000). Functional genes can also appear multiple times within bacterial genomes; for example the *amoA* gene is found two to three times per genome in beta-proteobacterial ammonia oxidizers but in single copies in the genomes of gamma-proteobacterial ammonia oxidizers (Norton et al. 2002).

Another limitation to consider is the fact that all DNA-based approaches, including qPCR, are dependent on DNA extraction from soil, which has the potential to introduce biases into the results. For example, all soil DNA extraction protocols lyse cells using processes such as enzymatic lysis, freeze–thaw, or bead beating. However, bacterial cells are not all lysed with the same efficiency by these various processes (Miller et al. 1999). So quantification of organisms using DNA-based approaches may be biased toward organisms that are lysed more easily.

Finally, for all DNA-based approaches it should be recognized that DNA can persist in soil outside of a cell for significant periods, from months to years

(Demaneche et al. 2001). For example, free extracellular DNA can bind to soil particles including clays and this binding can protect DNA from degradation by nucleases (Blum et al. 1997). Significant amounts of this extracellular DNA can be detected in soils (Demaneche et al. 2001) and can be coextracted with DNA from cells lysed by the DNA isolation procedure (Frotegard et al. 1999). Thus, when working with environmental samples, it is possible for PCR to amplify DNA from cells that are no longer living, which would be a confounding factor in enumeration of bacterial populations.

2.4.4 Expression of Population Density per Unit of Soil

The ultimate goal for most ecological studies is to compare data from divergent soil ecosystems and to evaluate the impact of differences in physical, chemical, or biological properties between the sites on the activities. Traditionally, soil population densities, enzymatic activities, as well as chemical concentrations have been expressed on a per gram of dry soil basis. This method of data presentation may be reasonable with some systems, but with the diversity of soils encountered in field studies, consideration of the bulk density and the volumes of soil represented is important. This conclusion is based on the observation that, in reality, soil processes occur in a three dimensional system, i.e. a volume of soil. The quantity of soil contained within that volume is highly variable. The bulk densities of most mineral soils range from about 0.9 to 1.2. Thus, activity per gram dry soil is approximately the activity per cubic cm of soil. In contrast, bulk densities of soils containing high levels of organic matter or organic soils may be quite low. For example, organic soils frequently have bulk densities of 0.1 g soil cm^{-3} or less. Assuming a bulk density of 0.1, any activity expressed per gram of dry soil would represent 10 cm^3 of soil within the native environment. Thus, if results for the low bulk density organic soil were compared on a dry weight basis with those similarly expressed for an average mineral soil, a 10-fold error in conceptualization of the field situation would result from the different field volumes represented. Hence, it is important when evaluating published data, or preparing one's own data for publication, to include a study of the site soil bulk density so that activities or populations in comparable quantities of soil can be evaluated.

2.4.5 Products of Soil Autecological Research

Provision of a list of most frequently encountered bacterial or fungal species, historically the most commonly studied soil populations, can only be criticized for its omissions than for its inclusiveness. For not only is there a vast array of species naturally present and functioning within any soil ecosystem, but propagules gain entry by transport through air, water, or sediment transport. Further, a variety of anthropogenic activities (such as those associated with waste disposal) augment the

range of microbial species present. Commonly encountered soil bacterial species include representatives from the genera *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Caulobacter*, *Cellulomonas*, *Clostridium*, *Corynebacterium*, *Flavobacterium*, *Hyphomycrobium*, *Metallogenium*, *Micrococcus*, *Mycobacterium*, *Pseudomonas*, *Sarcina*, *Streptococcus*, and *Xanthomonas*. These heterotrophic bacterial genera are augmented in soil by autotrophic and mixotrophic representatives, including a variety of nitrifiers, *Thiobacillus* species, and iron bacteria. Actinomycete populations include the commonly encountered *Nocardia* sp. and *Streptomyces* sp. Fungal populations are composed of a variety of slow- and fast-growing species. Perhaps the most commonly encountered are *Penicillium* and *Aspergillus* species plus representatives of the *Zygomycetes* and the mycorrhizae associated ascomycetes and basidiomycetes.

The objectives of experiments requiring identification of soil populations range from isolation of individuals with traits of interest to elucidation of the diversity of soil microbes by identification of tens to hundreds of different microbial species. The latter studies include evaluation of the interactions between the diverse populations present in soil as well as a determination of the effects of external stimuli, including anthropogenic impacts such as clear-cutting of forests or industrial pollution, on microbial diversity. Diversity studies of soil microbes have been limited in the past due to the difficulty of determining function *in situ* and the vast quantities of data necessary to identify the species present or to group the organisms into guilds based on structural or physiological similarity. Development of automated techniques for culture characterization and data analysis has made such studies more practical (see Mills and Bell 1986; Holder-Franklin 1986; Holder Franklin and Tate 1986; Russek-Cohen and Colwell 1986). These experiments have provided an understanding of the genetic potential within a given ecosystem and an indication of the resilience of the system to external perturbation, i.e. the capacity for homeostatic stability. Some systems with low species diversity, such as soils receiving thermal hot spring outflows, appear to be reasonably stable, but the classic maxim has been that the greater the species diversity, the higher the probability that the community has the capacity to overcome intrusions such as influx of contaminants or pH or temperature alterations. Much of this stability is derived from latent genotypic capability. Methods for assessing soil microbial diversity will be discussed in more detail in Chapter 3.

2.5 Principles and Products of Synecological Research

Although the sciences of microbiology and ecology have traditionally involved association of a genus and species name with the living creatures studied, as indicated above, such activities have more limited value in soil ecosystem characterization.

This is due in part to problems defining bacterial species and the capacity of soil organisms to exist in active, inactive, or resting states. Thus, the presence of an organism does not necessarily prove its current participation in ecosystem driving or defining activities. Synecological studies are therefore of growing importance in defining soil activity. Synecological research involves assessments of metabolic activities, enzymatic activities, as well as the properties of the soil ecosystem resulting from and controlling these activities.

As with the evaluation of specific microbial populations discussed above, a prime concern in examining soil as a "tissue" is collection of a representative soil sample. Since soil enzyme analysis or respiration measurements generally require larger samples than are necessary for detection of individual microbial species (grams of soil for a synecological study vs. perhaps milligrams or less for an autecological analysis), sample heterogeneity difficulties are reduced. Composited soil samples collected in a manner to average system variability become more practical; for example, a composited soil sample from a corn field may contain equal quantities of soil from between rows and within rows. Considerations with soil collection may reside more with concern for aboveground plant distribution affect or soil horizon influences than is possible with autecological studies.

2.6 Interphase Between Study of Individual and Community Microbiology

Neither the study of individual species present in soil and their metabolic capability nor consideration of soil metabolic activities solely provides the conceptual basis upon which to construct the science of soil microbiology. Axenic cultures of soil microorganisms provide a detailed understanding of the nuances of the metabolic intricacy of the "players" in the soil ecosystem. Evaluation of enzymatic and respiratory activities of isolated soil samples demonstrate site-specific and perhaps even chronologically limited system traits, but without an understanding of the organisms present little is revealed regarding the resilience of the community. That is, both the axenic culture and the soil sample are informative for the soil microbiologist.

This conclusion is demonstrated quite clearly when the impact of soil heterogeneity on growth factor limitations and microbial interactions are considered. Microorganisms require growth factors (i.e. vitamins and amino acids) for growth. A greater proportion of soil bacteria require these factors than are capable of synthesizing their metabolic needs *de novo*. The effect of this observation can be seen by examining a hypothetical reaction catalyzed by a microorganism requiring a vitamin. Assume that in the test tube the following reaction sequence is catalyzed by a single microbe:

$$A \rightarrow B \rightarrow C \rightarrow D$$

Let us hypothesize that for this reaction to go to completion a growth factor is required for conversion of product B to product C. This may not be considered in laboratory experimentation because the growth media typically used are supplemented with yeast extract or comparable vitamin and amino acid sources to provide all nondefined growth needs. Thus, experimental results are published with the conclusion that the bacterial isolate, perhaps one commonly occurring in soil, is perfectly capable of mineralizing the compound of concern, yielding carbon dioxide and water. That is, the compound of interest is concluded to be biodegradable. Unfortunately, the requisite growth factor may be missing from the soil microsite. Therefore, instead of observing complete mineralization as the laboratory data would have predicted, product B accumulates. Thus, a substance that laboratory data indicated is biodegradable yields intermediates which accumulate in the ecosystem.

Laboratory data could also predict environmental stability of a product when complete decomposition is observed in native soils. This situation occurs in ecosystems where no single microbial species possess the enzymatic capacity to convert the substance to carbon dioxide and water, but a consortia exists which can catalyze the conversion. Again, consider the above generic reaction sequence:

$$A \rightarrow B \rightarrow C \rightarrow D$$

In this situation, substance A may be converted to product B by a one microbial species. A second or even a third microorganism could be postulated to be required for transformation of products B–C and C–D. Thus, data from axenic culture indicates environmental stability of substance A whereas amendment of soil with substance A results in complete conversion of substance A to product D.

2.7 Concluding Comments

This analysis of the soil biological community provides justification for reaching a seemingly heretical conclusion that the total potential of a soil ecosystem exceeds the sum of its parts. Conceptually, soil is readily separated into a mass of mineral and chemical components as discussed in Chapter 1 plus a variety of living organisms. It is these latter participants in the system that transform the soil mineral matrix into the ever changing, life supporting substance essential for our terrestrial existence. Although a vast array of biological processes occur in a soil sample, a much greater potential is contained within the unexpressed microbial metagenome. Thus, development of an understanding of the intricacies of the interaction of the biological dimension of soil with its physical and chemical aspects is essential for proper stewardship of our ecosystem. This conceptual understanding is based on the results of a variety of autecological and synecological studies of soil

organisms and processes. As introduced in the brief overview of some methods available for analysis of soil microbes, validity and interpretation of the data collected in these ecological studies must be predicated on a firm appreciation of the methodological problems associated with soil microbiological research. Even with their inherent limitations, such studies provide the foundation for further incursions into evaluation of the ever-changing world of the soil microbe.

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Microbial Diversity of Soil Ecosystems

Soil ecosystems include a diverse array of organisms, ranging from macroorganisms, such as plants and animals, to microorganisms, such as bacteria, archaea, and fungi. In most natural ecosystems each of these groups is composed of a range of species and thus contributes to the overall biological diversity of the system. Some notable exceptions to this principle are the plant monocultures created by modern agriculture, and the monocultures that can occur due to the invasion of habitats by some exotic plant species (e.g. Thompson et al. 1987). The interacting macro- and microorganisms within soils have significant impacts on the nature of the soil ecosystem and the dynamics of the biological processes occurring therein. Therefore, the diversity of all of these classes of organisms can have significant implications for ecosystem function, stability, and resilience. The value of diversity to biological communities is exemplified by the increased pathogen susceptibility of plant monocultures (Leonard and Czochor 1980). Thus there is value in exploring and understanding the diversity of all of the classes of organisms found within soil.

Within soil ecosystems the diversity present within the microbial communities far outstrips the diversity of even the most diverse communities of macroorganisms. For example, typical bacterial cell densities in soil range from 10^8 to 10^9 cells per gram of dry soil; DNA reassociation analysis has shown that a single gram of soil contains 4000–7000 unique bacterial genomes (Torsvik et al. 1990). Many questions remain about the depth and significance of this diversity, as methodological constraints have made it virtually impossible to completely assess all of the bacterial diversity within a typical soil. Due to these methodological limitations the vast majority of bacterial diversity and the metabolic potential within these genomes have yet to be discovered. Also significant is the fact that the species composition of soil bacterial communities seems especially responsive to ecosystem changes and environmental perturbations, making soil bacterial diversity a potentially useful bioindicator of soil quality (Hartmann and Widmer 2006). For these reasons there has been and continues to be a great deal of interest in exploring and

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understanding soil microbial diversity. The objective of this chapter is to review some of the most commonly used methods for assessing soil microbial diversity, to discuss the limitations of the various methods, and to elucidate the types of information that are and are not provided by assessments of soil microbial diversity.

3.1 Classical Culture-Based Studies of Soil Microbial Diversity

Early studies of soil bacterial diversity involved isolating and purifying the bacterial species occurring in the soil of interest via culture-based methods and classifying the isolates according to species based on phenotypical characteristics. This approach can be used to assemble soil bacterial community surveys and to compare the species composition of communities from different sites or different experimental treatments. Various numerical approaches can be used to quantify diversity and assess differences in composition between multiple communities based on these culture-based surveys. For example, the diversity of a community can be quantified through the calculation of a diversity score based on one of several diversity indices such as the Shannon index (Shannon 2001). The composition of multiple communities can also be compared by calculating similarity scores for each pair of communities via a variety of similarity indices and performing a cluster analysis (see Russek-Cohen and Colwell (1986) for a review of this topic).

3.1.1 Value of Culture-Based Studies of Soil Microbial Diversity

Culture-based studies are relatively inexpensive to conduct and do not require advanced analytical equipment. For these reasons standardized protocols can be used throughout the world. Another major advantage of culture-based studies is that the organisms that have been isolated can be maintained in the laboratory and used to address additional research questions. For example, the specific biochemical composition and metabolic capabilities of organisms of interest can be analyzed for microorganisms that have been isolated in pure culture. Finally, with a culture-based approach the investigator can be sure that the organisms that have been isolated are viable, i.e. capable of growth.

3.1.2 Limitations of Culture-Based Studies of Soil Microbial Diversity

Culture-based studies are extremely time consuming and labor intensive in that large numbers of bacterial strains must be isolated from soil and characterized physiologically, which limits the depth to which a given community can be characterized.

Another of the main challenges associated with culture-based approaches to assessing diversity is that less than 1% of the microbes occurring in soil can be grown on standard laboratory media. Thus, the organisms isolated for the diversity characterization are a minor subset of the organisms existing *in situ*. This limitation could be acceptable if the isolated organisms represented a truly random and unbiased sample of the total bacterial community in the soils, or at least of the dominant populations. Unfortunately, this is clearly not the case, as the cultivation procedure by definition selects for certain organisms. Specifically, the cultivated organisms are those that can utilize the energy source provided in the medium under the physical and chemical limitations of the growth medium and incubation conditions. Furthermore, the organisms generally selected for study are those that grow rapidly on the isolation medium. Thus, slow-growing microbes or those that produce small colonies are commonly overlooked. Therefore, all that can be said about the strains isolated in a culture-based survey is that propagules of these strains existed in the soil system, but they may or may not have been active *in situ* (in surface soils, most of the microbes are inactive or are in a resting state at any given time), and they may or may not have been among the numerically dominant populations (i.e. other species or strains may have been numerically dominant but incapable of growth on the media used to select the bacterial isolates). Thus, the only conclusion that can be made is that the organisms isolated from the soil samples are the numerically dominant strains *that could be cultivated*.

3.1.3 The Challenge of Defining Bacterial Species

Another inherent difficulty associated with culture-based studies, and indeed with all studies based on species identification of soil bacteria, is the difficulty of defining a bacterial species. Bacterial species can be loosely defined as a collection of closely related bacterial strains. Classically, the strains forming a particular bacterial species have been grouped based upon possession of a series of common physiological capabilities, structural properties, and miscellaneous traits – such as motility and growth characteristics (e.g. pH or temperature tolerance ranges). A small percentage of the potential "species" in the bacterial world have been classified by this approach. Of greater concern to these definitions is that the entity that might be classed as a particular bacterial species is mutable. That is, its basic properties change. Mutations or transfers of genetic material from one species to another can readily blur the distinctions that were originally used to define the individual species. Thus, species definitions in bacteria are rather blurred and are to a large degree artificial.

Arguably, it could be stated that specific species designations are of greater significance when considering public health problems when evaluating soil populations. This statement is based on the observation that it is the overall metabolic capability

of the community and degree of similarity of the individuals that define the functional soil community, rather than the presence of nonchanging individual bacterial species – because they simply do not exist in the complex soil community.

3.1.4 Alternatives to Bacterial Strain Isolation

Ideally, a method for analysis of soil microbial diversity should involve evaluation of the total population. The method should include those species that grow on laboratory media and those that don't; those species that have been described as known species and those that possibly never will be; and those species that are active in the community as it currently exists plus those that have the potential to become active when conditions change. Since the simple task of culturing all members of the total microbial community from soil is insurmountable, a reasonable compromise to achieve this ideal objective would be to use a surrogate to assess microbial diversity in soil. This surrogate should be associated with living cells only, vary in a meaningful way in relationship to the overall microbial diversity in the system, and be sufficiently variable that differences in overall microbial diversity between communities could be detected. Candidates for use as surrogates in estimating soil microbial diversity include community metabolic (physiological) capabilities and various molecular components of cells. These will be discussed in more detail below.

3.2 Surrogate Measures of Soil Microbial Diversity

Fundamentally, to be aligned with classical considerations of biological diversity, surrogate measures of soil microbial diversity should be based on a delineation that varies in parallel with actual species diversity. Furthermore, the population sampling of the system of interest should either be all inclusive or at least fully representative of the diversity of the community. Few systems exist, either above or below ground, wherein enumeration of the totality of microbial species present is possible, so a means of assuring that those species detected are a truly representative subsample of the community is necessary. However, as the foregoing discussion of bacterial isolation and species characterization has indicated, these requirements present significant challenges for analyses of microbial diversity. Therefore, an estimate of the biological diversity of a soil microbial community must be based on selection of a surrogate that meets the above specifications of an ideal measure as closely as possible.

Further characteristics of an ideal surrogate measure of biological diversity are of a more practical nature and are derived primarily from two factors. First, the medium itself, soil, is extremely complex and heterogeneous. Second, there are a large number of factors that may significantly alter the soil community composition

with time. Therefore, the procedure should be quick and easy to conduct so that a large number of samples can be processed in a short period of time. This requirement is likely one of the largest impediments to the use of culture-based methods to measure microbial diversity. Ideally the method should also be reasonably economical to conduct and require only moderately specialized equipment and skill so that it can be generally applied to the study of the world's soil ecosystems. As with the more scientifically based specifications discussed above, these requirements are only partially fulfilled by methods currently available.

The procedures most commonly used to estimate soil biological diversity are community-level physiological profiling (CLPP), phospholipid fatty acid (PLFA) analysis, and a variety of DNA-based procedures. For each of these techniques, the nature of the soil biological variable assessed, the potential for provision of useful information about community structural variation in soil systems, and the limitations of the data will be discussed below.

3.3 Diversity Surrogates: Physiological Profiling

Bacterial species vary in many aspects of their physiology, including their sources of carbon and energy and their tolerances for a range of environmental factors (e.g. temperature, pH, salinity). One way in which bacterial species can vary is in their ability to utilize different carbon substrates, and this ability is one characteristic that can be used to identify and type bacterial isolates. One example of this is a tool known as BIOLOG (Miller and Rhoden 1991). The BIOLOG assay consists of a 96-well microtiter plate in which 95 of the wells each contain a different carbon substrate, and all of the wells contain a tetrazolium dye that undergoes a color change when it is reduced. During the assay a microbial suspension is added to all the wells within a plate and oxidation of individual carbon substrates triggers reduction of the tetrazolium dye. The resultant color formation in the well can be detected and quantified spectrophotometrically with a microplate reader. The BIOLOG assay was developed for the discrimination and identification of axenic bacterial and fungal cultures based on the pattern of oxidation of the substrates within the BIOLOG plates. There are several variations of the BIOLOG plates (e.g. gram positive, gram negative, fungal) each of which contains a different set of 95 substrates chosen for their ability to discriminate among species within the target microbial group.

3.3.1 Physiological Profiling of Isolates

The BIOLOG assay is effective at identifying axenic cultures (Klingler et al. 1992; Miller and Rhoden 1991), and so it can be useful for the identification of isolates in a culture-based assessment of soil microbial diversity as described above. Specifically, individual strains can be isolated in pure culture, and the BIOLOG

assay can be used to identify these isolated strains (e.g. Behrendt et al. 1997; Timonen et al. 1998). However, this approach still suffers from the limitations discussed above in regard to the use of any culture-based approach that requires isolation of bacterial strains from soil.

3.3.2 Community-Level Physiological Profiling

To avoid the limitations inherent in isolating individual bacterial strains from soil, some researchers have taken the approach of assessing the metabolic capabilities of whole microbial communities as a surrogate for diversity (Garland and Mills 1991). The use of the metabolic capabilities of a whole community as an indicator of microbial diversity is predicated on the assumption that significant differences in this parameter occur between soil samples that differ in microbial community composition. The truth of this assumption is not obvious. First, the prime energy source for soil ecosystems is photosynthetically fixed carbon. Second, although there are differences in the biochemical make-up of different plant species or of the same plant species growing under varying conditions, the general array of biochemicals encountered by soil microbes is not highly different from ecosystem to ecosystem. Therefore, soil microbes are facing a reasonably common group of biochemicals of varying concentrations in most ecosystems. It could thus be anticipated that all soil microbial communities would possess a reasonably similar arsenal of metabolic capacities to recover energy and nutrients from these substances. Therefore, variations in metabolic diversity between soils could be predicted to be minimal. Fortunately, in practice metabolic diversity does provide information that varies with ecosystem type and the conditions of particular ecosystems.

The metabolic capabilities of microbial communities have frequently been assessed using a variation of the BIOLOG assay in which soil extracts are directly inoculated into the wells of the BIOLOG plates, without any isolation of individual microbial species, in order to determine which substrates can be utilized by the whole microbial community. This technique has been referred to as a CLPP (Garland and Mills 1991). It has been found to provide reproducible profiles of model bacterial communities (e.g. Haack et al. 1995) as well as reproducible metabolic diversity patterns for soil ecosystems under a variety of cropping conditions and plant types (Bossio and Scow 1995; Garland and Mills 1991; Lupwayi et al. 1998; Winding 1994; Zak et al. 1994). CLPP has also been used to assess changes in microbial community composition caused by heavy metal contamination (Kelly and Tate 1998; Knight et al. 1997), variations in agricultural management of soils (Bossio and Skow 1998), variations in soil moisture (Zak et al. 1994), plant species variation (e.g. Ellis et al. 1995; Garland 1996a,b; Grayston et al. 1998) and the presence of chemical pollutants (e.g. Fuller et al. 1997; Thompson et al. 1999).

In the studies discussed above CLPP profiles were generated using one of the BIOLOG plates designed for identification of a specific microbial group (e.g. gram-positive bacteria, gram-negative bacteria, or fungi). Most commonly the plates designed for gram-negative bacteria were used. These plates are not ideal for community-level analysis because the substrates were chosen for their ability to discriminate species in pure culture, not for their environmental relevance. In addition, there was some concern that there was no interplate replication of the substrates, which limited statistical analysis of the data. In response to the interest in using the BIOLOG technology for CLPP profiling, BIOLOG developed the EcoPlate which contains 31 substrates that were selected and optimized from past environmental applications and are arranged in triplicate on each of the plates. Many recent studies have utilized the EcoPlates for CLPP profiling of field soils (e.g. Aira et al. 2000; Fontúrbela et al. 2012; Garau et al. 2007; Rutgers et al. 2016).

3.3.3 Value of Community-Level Physiological Profiling

CLPP has been shown to differ for bacterial communities with distinct species composition (Haack et al. 1995), and, as reviewed above, a number of studies have shown that CLPP generates reproducible profiles for microbial communities from a variety of soil types. Therefore, it is reasonable to consider CLPP as a useful tool for the determination of differences or changes in the composition of soil microbial communities. The diversity of substrates oxidized by a community in the CLPP assay can be quantified by using the CLPP data to calculate a diversity index, such as the Shannon index. The CLPP profiles of multiple communities can also be compared by multivariate techniques such as principal components analysis (PCA) (e.g. Kelly and Tate 1998; Kelly et al. 1999a) or multidimensional scaling (MDS) (e.g. Broughton and Gross 2000). CLPP analysis has some advantages over other methods for assessing diversity. For example, it is relatively inexpensive, requires a minimum of equipment (a microplate reader) and requires a minimum of training. Finally, the fact that the CLPP assay is based on measurement of metabolic processes, rather than being limited to species identification, may have some value for some research applications, as connecting species identification to functional capabilities can be a challenge for methods based strictly on species identification.

3.3.4 Limitations of Community Level Physiological Profiling

A primary question related to physiological profiling data is whether the patterns of metabolism of the individual substrates in the BIOLOG plate can be used to specifically describe how the various metabolic processes are occurring in the field. Generally they cannot. This conclusion is based mainly on the fact that the

conditions within the BIOLOG plates (e.g. pH, nutrient concentrations) and the incubation conditions used (e.g. temperature, light avilability) can be significantly different than what a community would experience in the field. In addition, the length of the CLPP assay (plates can be incubated from hours to days) provides ample opportunity for enzymes that were not expressed in the field to be expressed in the plates, and some evidence suggests that population changes can occur within the communities in the wells. For example, Smalla et al. (1998) used denaturing gradient gel electrophoresis and temperature gradient electrophoresis to demonstrate that significant changes in microbial community composition can occur within the wells during the CLPP assay. Therefore, it is reasonable to infer from a positive result for a substrate within the BIOLOG plate that the organism or organisms responsible for oxidation of that susbtrate existed in the soil sample, but no inference can be gained regarding the function of these organisms *in situ* since the environmental conditions may not have been appropriate for them to function in the soil. Conversely, a negative result for a substrate on the BIOLOG plate does not mean that organisms capable of oxidizing this substrate were absent from the soil, as these organisms may simply have been inactive under the conditions of the BIOLOG assay. Given these limitations, it is best to view the BIOLOG results as a "fingerprint" indicative of the potential metabolic capabilities of the community in question, and not as an indicator of metabolic processes actually occurring in the field.

3.4 Diversity Surrogates: Phospholipid Fatty Acid Analysis

A second alternative indicator of variation in microbial diversity in soils involves determination of the diversity of phospholipid fatty acids (PLFAs). PLFAs are the fatty acids that are structural components of the phospholipids that comprise cell membranes in eukaryotes and bacteria. The fatty acids found within cell membrane phospholipids can vary significantly in composition (e.g. chain length and degree of unsaturation), and there are consitent differences in PLFA composition across some microbial groups.

3.4.1 PLFA Analysis of Isolates

PLFA analysis has been used to characterize axenically grown bacterial isolates from soil for assessment of microbial community diversity (e.g. Germida et al. 1998), but this approach still suffers from the limitations discussed above in regards to the use of any culture-based approach that requires isolation of bacterial strains from soil.

3.4.2 Community PLFA Analysis

A more meaningful characterization of soil community dynamics can be attained by extracting phospholipids directly from a soil sample. For this approach it is assumed that all phospholipids contained within cells in the soil are equally accessible to the extracting agent, and that the PLFA profile of the extract is representative of the total PLFA content of the soil microbial community. For community PLFA analysis, phospholipids are extracted from a soil sample with solvent mixtures such as chloroform, methanol, and phosphate buffer (see White et al. (1979) for a detailed description of the extraction procedure). Following extraction, the chloroform fraction is concentrated and the phospholipids are separated on a silicic acid column. (see method of King et al. (1977)). The polar lipid fraction is collected, dried under nitrogen, saponified, and methylated. The resultant fatty acid methyl esters (FAMEs) are then identified via gas chromatography (e.g. with the MIDI protocol [MIDI 1995]). The MIDI system identifies and quantifies fatty acids of 9–20 carbon chain lengths.

The nomenclature for the fatty acids (e.g. 16 : 1 ω7t) refers to the total number of carbon atoms in the chain: the number of double bonds, the position of the double bond(s) from the methyl end of the chain (indicated by the number after the ω), and the conformation (cis vs. trans). Different subgroups of microorganisms (e.g. fungi, mycorrhizal fungi, gram-positive bacteria, actinomycetes, gram-negative bacteria) are represented in the profile by the quantities of specific fatty acids. For example 16 : 1 ω5c is an indicator for arbuscular mycorrhizal fungi (Haack et al. 1995), 16 : 0 10 for nocardioform actinomycetes, TBSA 18 : 0 10 Me and 17 : 0 10 Me for actinomycetes (Frostegård et al. 1993), 18 : 2 ω6c for fungi (Guckert et al. 1985), 15 : 0iso, 16 : 0iso, and 10Me16 : 0 for gram-positive bacteria (Zak et al. 1996), and 16 : 1w7c, cy17 : 0, 18 : 1w7c, and cy19 : 0 for gram-negative bacteria (Zak et al. 1996). Thus, the resultant phospholipid fatty acid profile provides an indication not only of changes in the composition of the microbial community (by changes in the overall composition of the profile) but also of the changes in the relative abundances of several broad groups of microbes. This procedure has been shown to be a sensitive indicator of shifts in soil microbial community composition resulting from human impacts, such as heavy metal contamination (Frostegård et al. 1996; Kelly et al. 1999a; Pennanen et al. 1996), contamination by organic pollutants (Wang et al. 2016), soil restoration (Kelly et al. 1999b), and increased atmospheric carbon dioxide (Janus et al. 2005). The method is sufficiently sensitive that impacts of root herbivory on rhizosphere communities (Denton et al. 1999) as well as shifts in community composition due to soil management (Bardgett et al. 1997; Bossio et al. 1998; Frostegård et al. 1993; Klamer and Bååth 1998; Kelly et al. 2007) have also been detected. A useful adjunct to the analysis of phospholipid fatty acid diversity is that the total phospholipid fatty acid content of the extract can

be used to estimate total soil microbial biomass (e.g. Denton et al. 1999; Janus et al. 2005; Kelly et al. 2007; Klamer and Bååth 1998).

3.4.3 Value of PLFA Analysis

PLFA analysis is an effective tool for the comparison of microbial diversity between samples. PLFA generates quantitative data on the relative abundance of specific PLFAs, and as was described above for the CLPP assay, PLFA data can be used in the calculation of diversity indices, such as the Shannon index. PLFA data can also be used to assess the similarity of communities via multivariate techniques such as PCA (e.g. Kelly et al. 1999b) or MDS (e.g. Baniulyte et al. 2009; Broughton and Gross 2000; Kelly et al. 2007). Because PLFA is a relatively inexpensive procedure, with the main disposable cost being the solvents used in the phospholipid extraction, large numbers of samples can be run in parallel and compared. These factors can allow for higher degrees of replication and more complex experimental designs than might be possible with more expensive methods. Indicator PLFAs (discussed above) also enable discrimination of some broad microbial groups (e.g. fungi, gram-positive bacteria, gram-negative bacteria). The ability to assess relative abundance of both bacteria and fungi in a single assay is a unique feature and a major advantage of the PLFA assay.

3.4.4 Limitations of PFLA Analysis

The main limitation of the PLFA procedure is that it does not have a very high level of taxonomic resolution. This assay can provide insight into the relative abundances of broad microbial groups (e.g. fungi, gram-positive bacteria, gram-negative bacteria), but it cannot approach the level of resolution needed to identify bacterial species. Another concern is that there is not a simple, direct correlation between the abundance of a particular PLFA and cell number. Cellular fatty acid composition can vary between bacterial species, and individual bacterial species can alter the fatty acid composition of their cell membranes in response to environmental factors such as temperature (e.g. Könneke and Widdel 2003). Therefore, while increases or decreases in certain indicator fatty acids can be inferred to represent increases or decreases in the abundance of the microbial group linked to that fatty acid, care should be exercised in linking PLFA abundance to cell abundance.

3.5 Nucleic Acid-Based Analyses of Soil Microbial Diversity

Nucleic acids represent another potential surrogate for the analysis of soil microbial diversity. The genomes of all cells are composed of DNA, and variations in DNA sequences can be used to assess microbial community composition and

diversity with a level of resolution far greater than any of the other surrogates discussed above. A variety of genes or genomic regions can be used for the analysis of soil microbial diversity, but the most popular is the small subunit ribosomal RNA (SSU rRNA) gene, which was first proposed as a phylogenetic marker by Carl Woese in 1977 (Fox et al. 1977). The advantages of the SSU rRNA gene as a phylogenetic marker are that (i) it is present in all cells, (ii) its sequence is fairly well conserved across phylogenetic groups, allowing routine comparisons of sequences across these groups, and (iii) there is enough variation within the gene to infer phylogenetic relationships between taxonomic groups. Based on these characteristics, SSU rRNA gene sequences have been used to develop a comprehensive phylogenetic framework for all organisms (Woese et al. 1990), and these gene sequences are routinely used for the identification and phylognetic classification of microorganisms. Molecular techniques that analyze prokaryotic SSU rRNA genes, specifically the 16S rRNA genes, have been widely used for the analysis of soil microbial diversity (Macrae 2000).

3.5.1 Nucleic Acid Based Analysis of Isolates

As with the other diversity surrogates discussed above, nucleic acid-based tools can be applied to prokaryotic organisms isolated from soil. Specifically, DNA can be extracted from a pure culture isolated from soil, the 16S rRNA gene of the isolate can be amplified and sequenced, and the isolate can be identified by comparing its 16S rRNA gene sequence to those in one of several large databases, such as GenBank (www.ncbi.nlm.nih.gov/genbank) or the Ribosomal Database Project (https://rdp.cme.msu.edu). Comparison with these databases will generally enable identification of an isolate to a high level of taxonomic resolution, often to the species or strain level, although these identifications are limited by the extent to which the databases cover the diversity present within soil microbial communities. Identification of soil isolates by DNA sequencing has become a standard method for strain identification and characterization, but as a tool for assessing soil microbial community diversity, DNA sequencing of strains isolated from soil has all of the limitations discussed above with respect to the inherent difficulty in culturing environmental microorganisms in the lab.

3.5.2 Community Nucleic Acid Analysis

Analogous to the approach discussed above for the PLFA assay, an alternative to a culture-based application of DNA sequencing is a culture-independent approach in which DNA from an entire microbial community is extracted directly from a soil sample and analyzed. There are multiple methods that have been used to extract DNA directly from soil samples, and the goal of all of these methods is to isolate DNA from the microbial community in an unbiased way, such that the

DNA extracted will be representative of all of the taxa within the community, and to purify this DNA adequately to enable downstream molecular analysis.

3.5.3 DNA Extraction

Methods used to extract community DNA directly from soil are designed to accomplish the following tasks: break open cells to release DNA, protect DNA from degradation, and separate DNA from the other components of the soil sample. A variety of methods to accomplish these tasks have been published, and some are better suited to certain soil types than others (e.g. see Braid et al. 2003; Moyer et al. 1996; Tebbe and Vahjen 1993; Tsai and Olson 1992; Zhou et al. 1996). The methods also vary in terms of the equipment needed and the use of hazardous chemicals. Due to the popularity of DNA methods for analyzing microbial communities, several companies have developed commercially available DNA extraction kits, many of which are specialized for different sample types (e.g. soil, water, or fecal samples). Many comparisons of various DNA extraction methods can be found in the literature (e.g. Stach et al. 2001). Researchers should be careful in selecting a DNA extraction approach that is appropriate for the soil types they wish to analyze.

Analysis of soil DNA extracts is complicated by the heterogeneity of the chemical components found in soil and the potential for some of these chemicals to interfere with the extraction or subsequent analysis of the DNA. A primary concern is the potential for interference in DNA analytical procedures by humic acids that can co-extract with DNA. Thus, the extraction method selected must either minimize co-extraction of humic acids or involve a purification step to remove the humic acid fraction. Commercially available kits for DNA extraction from soil generally include purification steps to limit co-extraction of humic acids.

Another concern associated with DNA extraction from soils is the potential for the extraction to introduce bias into the analysis if DNA is not isolated from all cell types equally, and multiple studies have suggested that DNA extraction can introduce bias into analyses of soil microbial community composition (e.g. Carrigg et al. 2007; Feinstein et al. 2009; Martin-Laurent et al. 2001). Researchers applying these methods should be cognizant of the issue of extraction bias when interpreting results.

Finally, a key assumption of techniques based on analysis of community DNA extracted from soils is that the DNA has been extracted from living cells. Recent evidence has shown that DNA can persist in soils for significant periods of time by binding to soil particles and that this binding can protect free DNA in soils from DNase degradation. Thus, it is possible that some of the DNA extracted from soil could actually have come from cells that are no longer living in the soil being analyzed. The detection of DNA from dead cells, refered to as “relic DNA,” can be a

confounding factor in studies of microbial community structure (Carini et al. 2017). Therefore, both of these limitations, extraction bias and DNA persistence in soils, should be considered when interpreting results from community DNA-based analyses.

3.5.4 Analysis of Community DNA

DNA extracted directly from soil samples can be analyzed by a wide array of different techniques. The most widely used require amplification of target genes (e.g. 16S rRNA genes) from community DNA via the polymerase chain reaction (PCR). PCR-based techniques include clone library sequencing, fingerprinting techniques such as T-RFLP and denaturing gradient gel electrophoresis (DGGE), and more recently a variety of high-throughput amplicon sequencing approaches. There are also a number of techniques for the analysis of community DNA that do not require PCR amplification of target genes, including metagenomics. Each of these approaches will be discussed below.

3.6 PCR-Based Methods

PCR-based methods analyze single gene targets from within community DNA by amplifying target genes via PCR prior to downstream analyses. As described above, the 16S rRNA gene is the most common target for analyses of prokaryotic community diversity, but a variety of other genes have been used as well. For example, functional genes associated with specific metabolic pathways, e.g. genes encoding enzymes involved in processes such as ammonia oxidation, denitrification, and sulfate reduction, are also popular targets for the PCR-based methods described below.

3.6.1 Clone Library Sequencing

A phylogenetic survey can be conducted for a soil bacterial community by extracting total community DNA directly from a soil sample and amplifying the 16S rRNA genes from within the community DNA via PCR using universal primers that have been designed to target the 16S rRNA genes from all bacteria (e.g. Borneman et al. 1996). The amplicons produced are then inserted into cloning vectors (plasmids designed to carry PCR amplicons) and these cloning vectors are inserted into bacterial cells (often *Escherichia coli*) that have been pretreated (e.g. electroporation or chemical treatment) to enable plasmid uptake. This collection of cells containing amplified 16S rRNA genes within plasmid vectors is known as a clone library, and the 16S rRNA genes within this library can be sequenced to

produce a random survey of the 16S rRNA genes found within the original community. This technique is known as clone library sequencing. The sequenced genes can be assigned to taxa by comparison to Genbank or the RDP database (as described above) or they can be binned into operational taxonomic units (OTUs) based on degree of sequence similarity. The collection of taxa or OTUs within a clone library can then be used to calculate community diversity (e.g. Dunbar et al. 1999) or to compare communities via multivariate techniques such as PCA (e.g. Hur and Chun 2004) or MDS (e.g. Borneman et al. 1996).

3.6.1.1 Value and Limitations of Clone Library Sequencing

Clone library sequencing has significantly increased our understanding of microbial diversity by revealing much greater diversity than was detected by classical culture-based studies. However, clone library sequencing is time consuming, labor intensive, and expensive. Although the cost and time required for large scale sequencing projects are decreasing quickly, the incredible diversity of microbial communities makes it impractical at this point to attempt to sequence every amplicon produced from PCR amplification of an environmental sample. For example, soil from a beech forest showed total bacterial counts of 1.5×10^{10} cells g soil^{-1} and approximately 4000 unique bacterial genomes based on DNA reassociation analysis (Torsvik et al. 1990), and a more recent study used reassociation kinetics to determine that a pristine soil contained more than one million distinct genomes. Attempting to capture all of this biodiversity by sequencing a clone library is impractical. Therefore, although phylogenetic inventories of microbial communities based on cloning and sequencing 16S rRNA genes can provide useful information, these inventories will generally be incomplete, so populations comprising very small fractions of the overall community may not be detected. In addition, clone library sequencing is too slow to be useful for routine profiling and comparison of large numbers of environmental samples.

3.6.2 DNA-Based Fingerprinting Techniques

As an alternative to cloning and sequencing, techniques such as DGGE and terminal restriction fragment length polymorphism analysis (T-RFLP) can be used to analyze amplicons produced by PCR amplification of DNA extracted from an entire microbial community. These techniques generate profiles for microbial communities based on differences in the gene sequences of their constituents. Specifically, these techniques involve separation of amplicons on an acryamide electrophoresis gel. In DGGE the amplicons are separated based on sequence differences that lead to differences in the denaturing properties of the amplicons, and in T-RFLP the amplicons are separated based on sequence differences that lead to differences in the sizes of fragments produced after digestion of amplicons with

restriction enzymes. Both DGGE and T-RFLP produce patterns of bands on a gel, with the bands representing distinct genotypes within a community of organisms. The presence or absence of specific bands and the intensity of the bands produced from a community (i.e. the fingerprint) can be compared between different communities and can provide insight into differences in microbial population structure between different soil habitats or changes in soil microbial population structure over time or with different experimental treatments.

3.6.2.1 Value and Limitations of DNA-Based Fingerprinting Techniques

DNA based fingerprinting techniques, including DGGE and T-RFLP, can be effective tools for comparisons of microbial communities across sites or treatments, and they can both be used to determine if significant differences in community composition exist. Multivariate ordination techniques such as PCA (e.g. Janus et al. 2005) or MDS (e.g. Baniulyte et al. 2009) can be used with either DGGE or T-RFLP data to compare profiles. DGGE and T-RFLP can be used with any gene target, including genes used as phylogeneitc markers (e.g. 16S rRNA genes) or functional genes, as long as suitable primers exist. DGGE and T-RFLP both also have very high resolution, as they are able to discriminate amplicons differing by a single base. Finally, both DGGE and T-RFLP are relatively inexpensive to run, although they do require specialized equipment. However, DGGE and T-RFLP have two key limitations. The first is that it is not possible to identify the taxonomic affiliations of the bands within the profiles, or to determine their specific DNA sequences. So while these techniques can be used to determine if soil microbial communities are significantly different in composition, they cannot identify which specific taxa are differentially distributed across the communities. Second, both DGGE and T-RFLP significantly underestimate the total diversity of a community and are biased toward the most numerically dominant organisms within a community. Therefore, researchers should be aware of these limitations before selecting these techniques and should consider these limitations when intepreting their results.

3.6.3 High-Throughput Amplicon Sequencing

In the late 1990s, efforts to sequence the human genome led to the development of DNA sequencing technologies that were significantly higher throughput than the traditional Sanger sequencing approach. In 2005 a pyrosequencing-based system was introduced that provided a 100-fold increase in throughput (i.e. base reads per unit time) over Sanger sequencing (Margulies et al. 2005), and in 2006 Sogin et al. first demonstrated the use of this system for the simultaneous sequencing of a pool of 16S rRNA gene amplicons produced from an entire microbial community without the need to insert amplicons into cloning vectors (Sogin et al. 2006). In their study, Sogin et al. applied this approach to the analysis of marine

microbial communties, and showed that it could produce 100–1000-fold more sequences per sample than a traditional clone library approach (Sogin et al. 2006). These groundbreaking studies led to a revolution in the field of soil microbiology, enabling vastly more extensive analyses of soil microbial community composition and diversity than had been possible previously.

Another key innovation that was developed for high-throughput sequencing-based profiling of microbial communities was the use of barcoding. Barcodes are short DNA sequences (generally eight bases) that are designed *in silico* and attached to the 5′ ends of PCR primers, resulting in the integration of these barcodes into amplicons during the PCR reaction. If distinct barcodes are attached to amplicons from different samples, amplicons from multiple samples can be pooled and sequenced together, and the sequences from individual samples can later be separated based on these barcodes (Binladen et al. 2007). This barcoding strategy enables researchers to analyze large numbers of samples in parallel to whatever level of sequencing depth (i.e. number of sequences per sample) is desired.

Since the introduction of pyrosequencing, newer high-throughput sequencing platforms have continued to emerge, and microbiologists have applied these new platforms to the analysis of 16S rRNA gene amplicons. Examples include Illumina (Caporaso et al. 2010; Lazarevic et al. 2009), Ion Torrent (Brown et al. 2013; Whiteley et al. 2012) and PacBio (Mosher et al. 2013; Schloss et al. 2016). The common feature of these newer sequencing platforms (sometimes refered to as "next-generation sequencing") is that they provide much higher throughput than Sanger sequencing (i.e. more bases sequenced per unit time) at a much lower cost per base, but each of these platforms has specific advantages and disadvantges. Researchers interested in using one of these platforms to analyze soil microbial community diversity should carefully consider factors including cost, speed, read length, and accuracy.

The incredible increase in the amount of sequence data provided by high-throughput sequencing has revolutionized our understanding of microbial community diversity, but it has also necessitated the development of more advanced computational tools to analyze these incredibly large data sets. Specific tasks that must be accomplished for analysis of high-throughput sequencing data sets include separation of sequences by sample based on barcodes, removal of low quality sequences, alignment, binning, and taxonomic assignment of sequences based on comparison to available databases. Many researchers have developed computational tools to accomplish these individual tasks, but two comprehensive software packages have been developed specifically for the analysis of 16S rRNA gene amplicon libraries produced via high-throughput sequencing: mothur (Schloss et al. 2009) and QIIME (Caporaso et al. 2010). These two packages are freely available to researchers, and both are widely used in the field of soil

microbiology. Both mothur and QIIME can accomplish the basic tasks listed above, but they differ in the specific methods used to accomplish these tasks. Researchers interested in the analysis of 16S rRNA gene amplicon libraries produced via high-throughput sequencing are encouraged to explore both of these tools as possible options for data analysis.

3.6.4 Limitations of PCR-Based Methods

It should be noted that there are some significant limitations to techniques (such as those described above) that are based on PCR amplification of DNA extracted from soil. One assumption of these techniques is that the DNA from all organisms present in the sample will be amplified with the same efficiency. This assumption may not be correct in all cases, and in some cases the process may favor the amplification of DNA from certain organisms. Biases in amplification efficiency can be caused by differences in cell lysis or DNA extraction efficiency, differences in gene copy number within the organisms or differences in the efficiency of the PCR reaction itself. PCR bias can result in misrepresentation of phylogenetic diversity in phylogenetic surveys and T-RFLP and DGGE profiles, and thus PCR bia can be problematic in microbial ecological studies. Researchers applying PCR-based approaches to the analysis of soil microbial community diversity need to be cognizant of these limitations.

3.7 Metagenomics

Metagenomics can be defined as the simultaneous analysis of the genomes of a population of microorganisms (Handelsman 2004). Metagenomics seeks to analyze not only the taxonomic composition of microbial communities based on marker genes such as the 16S rRNA gene (as described above), but also to document the collection of genes present within an entire microbial community. Metagenomics does not require PCR amplification, so it avoids some of the limitations of PCR-based approaches that are described above.

The earliest metagenomic studies were based on direct isolation of community DNA from an environmental sample (as described above) followed by random insertion of fragments of this extracted DNA into cloning vectors and transformation of the clones into host bacterial cells. These metagenomic clone libraries were then either sequenced randomly in a process known as shotgun metagenomics (e.g. Tyson et al. 2004; Venter et al. 2004), or the clones were first screened for the presence of phylogenetic indicator genes (e.g. 16S rRNA genes) or for the presence of genes expressing specific functions (e.g. antibiotic resistance; Handelsman 2004) and then selected clones were sequenced. The first metagenomic studies

were conducted on marine samples (Stein et al. 1996), and metagenomic studies of soil microbial communities soon followed (Henne et al. 1999; Rondon et al. 2000). Metagenomic studies of soil microbial communities have provided a wealth of insights into the functional potential present within these communities, including the discovery of novel antibiotics (Gillespie et al. 2002), antibiotic resistance genes (Riesenfeld et al. 2004; Torres-Cortés et al. 2011), and degradative enzymes (Henne et al. 1999, 2000).

The earliest metagenomic studies (including those listed above) relied on Sanger sequencing of clones, and were thus limited by the capabilities of that sequencing platform. Advances in high-throughput sequencing technology (as discussed above) have enabled direct, untargeted sequencing of DNA isolated from environmental samples without the need for cloning. This approach has been applied to the analysis of soil microbial communities (e.g. Luo et al. 2014; Mackelprang et al. 2011; Yergeau et al. 2010). For high-throughput metagenomic studies, the DNA isolated from an environmental sample is first randomly fragmented, using either enzymatic or physical fragmentation, to produce a sequencing library, and this library is then sequenced using a high-throughput sequencing platform. The Illumina HiSeq platform is the most popular for metagenomic studies, due to its high output and high accuracy, but other technologies are beginning to be adopted as well (Quince et al. 2017). After sequence data are obtained and low-quality data removed, the first step involved in analysis is assembly of short reads into larger contigs. In some cases, including samples with low diversity and a high degree of sequencing depth, it may be possible to assemble contigs into complete bacterial genomes, but for most soil microbial communities, this will not be possible for the vast majory of the data obtained. In this case the assembled contigs can be compared to publically available databases to identify phylogenetic indicator genes (e.g. 16S rRNA genes) or genes associated with specific functions (e.g. antibiotic resistance) or metabolic pathways. Numerous approaches for computational analysis of metagenomic data are currently available, with new tools continuously being developed. The choice of appropriate tools and strategies can be challenging (see discussion of limitations below), but these choices should always be based on the goals of the study.

3.7.1 Limitations of Metagenomics

A key parameter in metagenomic analysis is the sequencing depth, i.e. the amount of base-pairs of data produced per sample. There is no simple answer to how much sequencing is enough, but the amount of sequencing depth required for a specific study will depend on the complexity of the community being analyzed and the questions being asked. In general, deeper sequencing will provide better coverage and higher liklelihood of retrieving sequences from low-abundance members of a community, but deeper sequencing increases costs significantly and creates challenges associated with analysis of massive data sets. Currently, microbiome studies

tend to generate between 1 and 10 Gb of data (Quince et al. 2017), but these values may change rapidly based on changes in sequencing technology and cost. At this time the costs for this level of sequencing are quite high and can limit access to this technique to well financed researchers. The high costs of metagenomic sequencing cannot also make it difficult for researchers to analyze enough samples and replicates to permit rigorous statistical analysis.

Metagenomic sequencing of complex microbial communities, such as those found in soils, generates unprecedented amounts of data, resulting in tremendous computational challenges. The power needed to analyze metagenomic sequence data sets far outstrips the capacity of even the most powerful desktop computers and generally requires the use of computing clusters, with many processors running in parallel. This type of computing power is expensive to buy and to manage, and thus computational needs can limit the ability of researchers at all but the most well-financed institutions to work with metagenomic data. Furthermore, there are no simple plug-and-play pipelines available for rigorous analysis of metagenomic data. Rather, significant expertise is required to navigate and apply the multitude of tools that are available for metagenomic data analysis, most of which must be run in Unix- or Linux-based systems. Therefore, the use of metagenomic sequencing for the analysis of complex microbial communities relies on the researchers either having advanced training in bioinformatics or collaborating with researchers with this expertise. Therefore, lack of appropriate expertise can also limit researchers' access to metagenomic tools.

3.8 Conclusions: Utility and Limitations of Diversity Analysis Procedures

A primary conclusion from our review of each of the major types of methods used to characterize soil microbial community diversity is that no single technique provides an all-inclusive, definitive description of soil microbial community diversity. As discussed above, each of the methods has specific advantages and disadvantages, and researchers need to be cognizant of these when choosing a method for their project and when interpreting results from a project. Moreover, the specific goals of the research project should always be the key factor in choosing a method for analyzing microbial community diversity, as no single tool is optimal for every job.

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Energy Transformations Supporting Growth and Survival of Soil Microbes

Along with the appropriate chemical and physical properties required for microbial survival, the soil habitat must contain the energy resources necessary for growth and sustainability of the microbes. Additionally, the microbial community must possess the genotypic traits required for energy recovery and be capable of expressing them *in situ* in the microbe's habitat. A simple question that could provide a starting place to examine energy transformations catalyzed by the resident microbes is "What is the ultimate product of microbial existence from the viewpoint of the microbe; that is, why is this energy supply essential?" A consideration of the microbial metabolic processes leads us to a rather simple, or perhaps, simplistic, response. The most important product from the view of life in soil is new microbial cells. Thus, the microbe must be capable of converting the mineral and organic components of its environment into the building blocks of microbial biomass. Some of the cellular components will be synthesized *de novo*, whereas others will be incorporated into cellular biomass with minimal or no alteration from the organic components of the microbes environment. All will require expenditure of energy. What is the primary energy source supporting the growth and function of the microbial community? It is reasonable to conclude that in the absence of major human-produced organic matter, the vast majority of the energy recovered by the microbial community is derived from oxidation of plant biomass. That is, the primary source of the energy "pipeline" used by the microbes is the sun.

Were this synthesis of new microbial biomass the sole outcome of microbial energy recovery, life on our planet would most likely be rather tenuous. True, the soil and the microbes contained therein are continually evolving. The biological, chemical, and physical components are continually changing – the microbes therefore must evolve in order to survive in the stressful soil system. This "dance of survival" performed by the growing microbes and their physical environment results in a multifaceted terrestrial ecosystem (Tate 1985, 1987).

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Before exploring the basis of microbial energy recovery, one further question requires attention. At this point, the primary energy source exploited by microbes has been elucidated, but the justification of the interaction with their physical environment must be at least a bit more complicated. The revelation that both the microbes and their habitat are constantly changing provides a window to understanding the significance of the complex energy recovery system to be described in this chapter. The microbial community is continually evolving to adjust to a soil environment that is also evolving in response to its every changing environment. But, change is only part of the story; the two components identified here must be interacting in a long-term, parallel interactive manner.

Are there examples of the physical and chemical changes in the physical properties of the microbial habitat resulting from the metabolic processes of the microbial population? Yes, the microbes (i) synthesize chelators and carbon dioxide that dissolve soil minerals thereby altering the nature and properties of soil minerals, (ii) mineralize and participate in synthesis of soil organic matter, thereby controlling soil aggregate formation and aboveground biomass production, and (iii) synthesize greenhouse gases such as carbon dioxide, methane, and nitrous oxides, thereby affecting terrestrial climate.

Based on these observations, a primary key to understanding microbial processes in soil is to understand the nuances of microbial energy recovery. Therefore, the basic growth and energy recovery mechanisms of the soil microbial community will be examined herein. This will be achieved by (i) evaluating the applicability of principles revealed through laboratory-based experiments to microbial growth kinetics in the soil ecosystem and (ii) elucidating the diversity of energy-yielding processes existent in soil microbial communities and how this array of microbial metabolic processes results in decomposition of both biologically and anthropogenically synthesized organic compounds.

4.1 Microbial Growth Kinetics in Soil

Since the primary benefits to microbes in soil are growth and/or maintenance of population densities, consideration of general microbial growth kinetics and specific examples of their replication and survival patterns in native soil ecosystems is a necessary starting point for analysis of soil physiological processes. To achieve this goal, the applicability of laboratory-derived relationships to field situations will be examined.

Open vs closed biological systems: In the laboratory, microbial growth kinetics are assessed as closed systems (e.g. culture flasks and test tubes, batch soil incubation chambers) or as open systems (e.g. a chemostat or in some primary aspects, soil columns) (Figure 4.1). A closed ecosystem is self-contained in that external

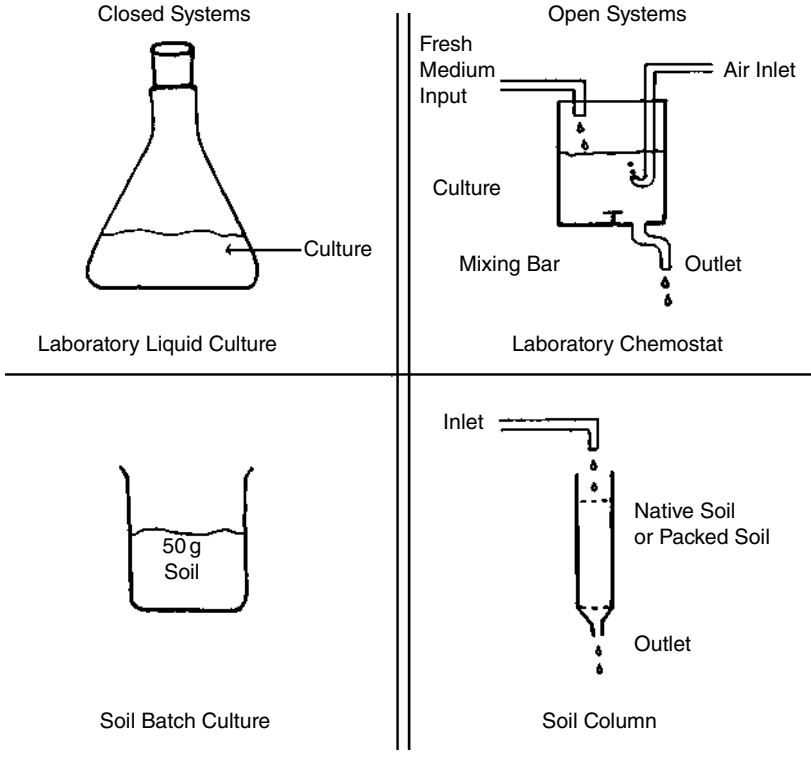

Figure 4.1 Examples of laboratory-incubated open and closed systems for study of microbial processes.

contributions to microbial function are excluded, whereas open systems receive inputs from processes occurring external to the defined boundaries of the site. For example, an open system can be exemplified by the recycling of organic wastes (sewage, biosolids, animal manures) on an agricultural soil. A consequence of the two defined situations is that since no external nutrient sources is provided in a closed system, energy and essential nutrients can become limited and metabolic waste materials or by-products can accumulate. In open systems, microbial cells can be constantly bathed in externally supplied growth supporting nutrients such as those provided by root exudates in the rhizosphere and metabolic by-products may not reach toxic levels in the vicinity of the colony or the individual cell. (In reality, all terrestrial ecosystems are open systems due to the input of photosynthetically fixed carbon driven by solar energy).

Although it is essentially impossible to exclude external influences from any portion of a soil site, conditions may exist locally where microbial population dynamics mimic those of a culture vessel incubated in the laboratory. This situation

can be exemplified by microbial community development in soil surrounding a portion of decaying insect biomass or plant debris. Growth of the microbial population is initially controlled locally by the quantities of readily metabolized substrates exuded from the animal or plant tissues. As the microbes grow and replicate, these easily metabolized substituents are depleted from the site or their concentrations may be reduced to growth-limiting concentrations. In the latter situation, cellular function is controlled by the rate of solubilization or release of the energy supplying nutrients from within the chitinous cell walls of the insect or the cellulosic walls of the plant biomass. In both cases, initially the more easily mineralized organic substrates are protected by the chitin containing structure of the insect or the cellulosic cell walls of the plant tissue. Eventually the energy-yielding substrates, e.g. sugars and proteins, are released by the action of enzymes such as chitinase or cellulose that catalyze the conversion of chitin or cellulose to easily metabolizable carbohydrates or amino sugars. As the cell wall structures are breached, the easily mineralized material contained within the cell is released. Upon depletion of all energy and nutrient supply pools in the vicinity of the microbial colony (i.e. total catabolism of the fixed carbon contained in the plant or animal debris), microbial growth ceases and the microbial population becomes quiescent. This state could be achieved prematurely, should growth-limiting cellular waste products accumulate to inhibitory levels prior to nutrient depletion, or if another essential nutrient supply is exhausted (e.g. free oxygen for aerobic microbes).

At this point, continued activity of the microbial population depends on an influx of nutrients from outside the boundaries of its habitats, as described above. A reasonable example of such a situation in field soils is comparison of a fallow field (noncropped) to an adjacent wheat field (Figure 4.2). In the fallow field, the microbial growth is limited by the existing soil organic matter pools with growth rates declining as the quantities of metabolizable organic matter declines whereas in the wheat field microbial metabolic activity and growth is controlled predominantly by the rate of influx of newly fixed carbon resulting from growth of the wheat crop.

The artificiality of this comparison of the native soil condition with closed cultural systems emerges when the totality of soil properties influencing microbial growth are considered. In the site described above, the primary delimiters of microbial replication were energy and nutrients, but imposition of moisture limitations, deficiencies in fixed nitrogen resources, or even difficulties with temperature modulation can easily be envisioned. Even with this small list of soil properties affecting microbial function, it is clear that both internal and external forces are generally involved in sustenance of the soil biological community. In a soil site contiguous with a variety of different soil habitats, stresses and the means of their relief for microbial populations may easily originate in regions beyond the artificial boundaries of an arbitrarily defined closed system. Thus, in soil, closed ecosystem properties may be invoked to simplify data interpretation, but the

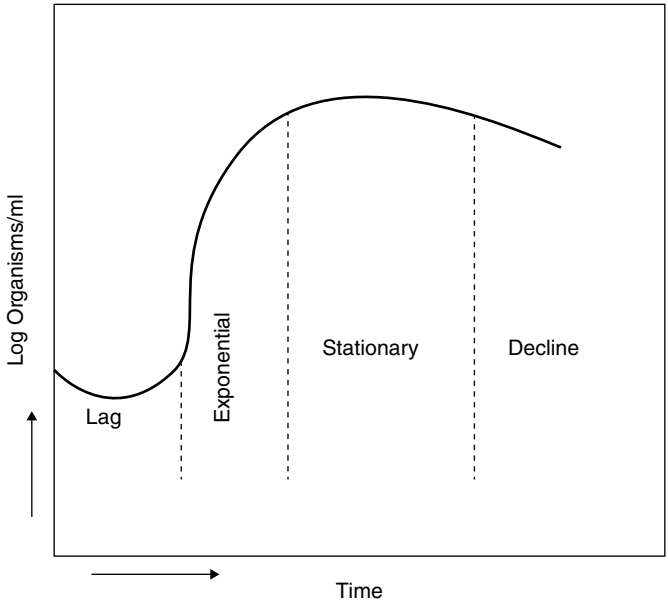

Figure 4.2 Idealized bacterial growth curve.

experimenter must always bear in mind that the microbes of interest are functioning in a system of interconnected and interacting ecosystem types.

Not only is a closed system an imperfect descriptor of the rates of biologically catalyzed processes in soils, but the soil ecosystem is also poorly described by the kinetics of an open system. (As will be shown below, chemostat systems are generally used to develop mathematical models of an open biological system.) Many delimiters of biological activity in soil are imposed from outside sources (e.g. nutrient and energy sources, moisture). This external control of microbial growth could result from a variety of conditions, such as from fixed carbon contributions of root exudates in rhizosphere soils, leaching of soluble fixed carbon sources from soil surface organic matter accumulations (e.g. forest litter layers, thatch accumulated in grassland soils), or anthropogenically created situations such as situations where industrial or domestic waste systems enter into a soil ecosystem (e.g. sewage or factory effluents). For example, overland flow of water from the cultivation portion of the wheat-fallow soil system depicted in Figure 4.2 could transport newly fixed carbon from the wheat field into the noncultivated portion of the site. A common occurrence of such transport is exemplified by the erosion of soil from the top or crest of the hill to the surrounding lowlands which can accelerate microbial growth and increase microbial biomass at the base of the hill due to metabolized organic carbon contained in the runoff water. Thus, it is reasonable to consider the impact

of nutrient availability in soils to that encountered in laboratory cultures where the growth of a microbial population results in reduction or even elimination of nutrient and energy reserves and imposition of growth-limiting conditions.

4.2 Microbial Growth Phases: Laboratory-Observed Microbial Growth Compared to Soil Population Dynamics

The intricacies of microbial growth and the impact of soil properties on growth rate are best exemplified by contrasting microbial growth principles derived from laboratory study to those occurring in the soil ecosystem. A idealized microbial growth curve (Figure 4.3) is commonly divided into four phases: lag (no observable growth), exponential growth (growth rate > death rate), stationary (growth rate approximately unchanging), and decline phase (death rate > growth rate). Each of these portions of the growth curve can be observed in soil ecosystems. It must be noted that major changes in population density generally occur only with an augmented input of easily metabolized organic matter. This pattern of population development is most commonly associated with soil sites where the native microbial growth rates

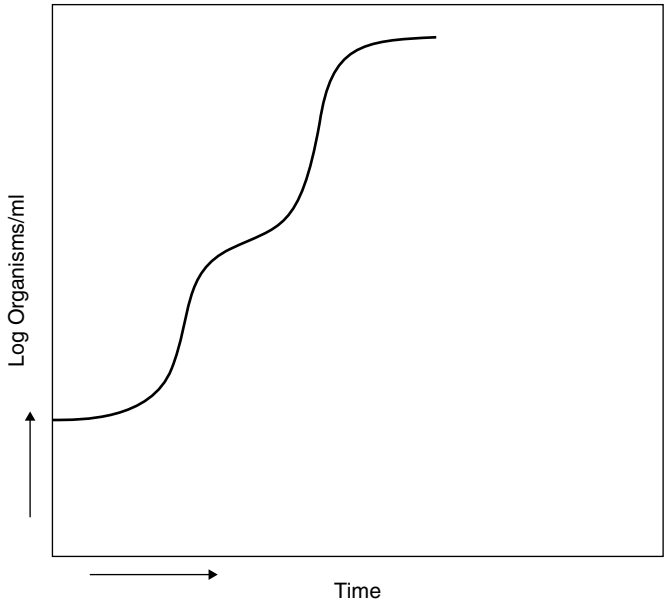

Figure 4.3 Bacterial growth curve resulting from sequential catabolism of two energy substrates (diauxic growth).

are stimulated by alteration of soil properties, such as introduction of plant debris into a carbon- or energy-limited community, through oxygenation of an anaerobic system where partially decomposed organic matter has accumulated, or through increased availability of native soil organic matter through disruption of a soil structure that precludes its catabolism. For example, conversion of native grasslands to wheat fields disrupts the soil aggregate structure protecting the soil organic matter results in major declines in soil organic matter pools. Once the previously existing impediments to microbial growth are removed, the cells replicate until a new limitation to community development is encountered (e.g. increased fixed nitrogen availability, decreased molecular oxygen levels).

Lag phase: A lag phase occurs when conditions are appropriate for microbial growth, yet there is a delay before a measurable change in microbial population density is detectable. This cellular division could be due to site amendment with metabolizable substances that were previously not present (e.g. pesticides, xenobiotic compounds) or alteration of the physical conditions (e.g. imposition of anaerobic conditions, which would stimulate growth of obligate anaerobes, or reduction of soil pH sufficiently to favor acidophilic populations).

In axenic cultures, a sensitive measure of cellular replication is usually employed. Thus, an accurate assessment of the lag time is gained. This assay sensitivity is difficult to achieve in soil. Most methods for quantifying soil microbial population density do not detect small changes in microbial population densities. Thus, several cell division cycles must occur before a measurable change in the microbial populations is detected. Therefore it could be concluded that at least a portion of the delays in response of soil populations due to system modification is the result of limitations in the methods used to quantify microbial population densities in soil.

Several methods are available for assessing increases in microbial biomass and biomass of individual microbial species in soil, including techniques for assessing the synthesis rates of various biomass specific compounds, such as lipids (^{14}C-acetate incorporation into microbial lipids (Barnhart and Vestal 1983; Federle et al. 1983; Tate 1985)) and nucleic acids (^{3}H-thymidine incorporation (Christensen et al. 1989)). Accuracy of the data yielded from these procedures is controlled by such factors as:

- the variability of biomass within soil samples
- the extraction efficiency for recovery of the population or cellular component assayed from the soil sample
- the minimal detection limit of the measurement procedure.

A variety of implications of the occurrence of a lag phase for microbial growth in soils on interpretation of data describing soil process rates can be conceived. A positive aspect of the occurrence of a lag phase in soil microbial growth kinetics is exemplified by considerations associated with use of biodegradable soil amendment, for

example, pesticides. Sufficient longevity of the pesticide in soil for it to interact with the target species is requisite. Greatest time is allowed if the population density of the microbes mineralizing (conversion of the organic substrate to mineral components, including carbon dioxide, ammonium, sulfate, and sulfide) of the soil amendment is limited. With populations of microbes capable of mineralizing the soil amendment of a few hundred or even thousands of organisms per g soil, several cell divisions may be required before detectable declines in the pesticide concentration occur. Thus, lag periods of days to weeks could be anticipated with more pristine systems. In such soil systems, the addition of the pesticide to the soil would have an adequate longevity to accomplish the desired ends before its equally desirable biodegradation occurs. Efficacy of repeated uses of the soil amendment in the system would be dependent on the longevity of the microbial populations involved in the decomposition of the pesticide.

A negative environmental implication of extended delays in biological decomposition of soil amendments is the potential for subsoil or groundwater contamination. Again, biodegradable compounds may be added to soil purposely or accidentally through mismanagement or through spills. To minimize the environmental impact in these situations, rapid biodegradation of the contaminant is necessary. The longer the lag in development of requisite biochemical activity, the greater the potential for leaching of the substance to a site where its presence is inappropriate and its decomposition is less likely to occur (for example, leaching of toxicants from the surface soil horizon to subsoil regions where decomposition is limited or groundwater contamination can occur). Similarly, overland flow of contaminated water or erosion of toxicant bound to soil particles becomes more problematic with longer longevity of the contaminant.

A practical example of the potential to exploit the occurrence of the lag phase is the collection of soil samples for estimation of *in situ* biochemical activities. Disruption of the soil physical structure during soil sampling necessarily causes sufficient changes in soil properties to stimulate microbial growth. For example, oxygen levels may increase as well as metabolizable carbon resources can be increased by the liberation of organic matter sequestered within the soil physical structure. The delay in growth (lag phase) and use of methods such as storage at 4 °C to reduce the microbial growth rate may afford the experimenter several hours to days to complete the laboratory assessments before statistically significant changes in population densities occur. The length of storage allowed before data are compromised by microbial growth depends upon the properties of the species of interest and the sensitivity of the assay method. The more sensitive the assay (i.e. the capacity to detect changes in population density of a few cells per gram soil) or the more prolific the microbes of interest (i.e. growth rates of a few hours under the storage conditions), the greater will be the problems associated with estimation of *in situ* soil microbial population densities. Less storage stability is anticipated for

samples to be assayed using polymerase chain reaction procedures or fluorescent antibody detection of microbial cells, since these methods are sufficiently sensitive to allow detection of increases of population densities of a few cells per g of soil. In contrast, considerable increase in populations quantified by most-probable-number procedures is possible before significant changes in data are demonstrable. Confidence limits of ±300% are not unusual with these types of procedures. In either case, a control study must be conducted to determine the acceptability of the chosen storage procedure for the soils and populations in question.

Exponential growth phase: Conceptually, occurrence of a period of exponential growth, a blooming *per se*, of specific microbial populations in soil is easily envisioned. Amendment of soil with essentially any biodegradable substance at a nontoxic, growth-supporting concentration results in an increase in the energy supply supporting microbial growth. The genetic diversity of the indigenous community as well as the commonly occurring immigration of propagules from adjacent as well as distant ecosystems (e.g. via wind and water erosion) virtually assure inoculation of the incorporated materials with organisms capable of the oxidizing them for energy and nutrients.

Other less appreciated, but perhaps more commonly occurring processes inducing microbes to enter an exponential growth phase are associated with indigenous ecosystem processes. Intrusion of predators, such as amoebae, nematodes, or earthworms, into a microsite where bacterial growth is controlled by space limitations may reduce the population densities sufficiently to allow several rounds of cell division. Indeed, balanced feeding of the predator could maintain the prey population in an active growth phase for extended time periods. When this occurs, the replicating microbial population could be dividing at a relatively rapid rate, but ingestion of the newly produced cells by the predators would prevent an observable increase in prey population density.

Similarly, a burst of cell division occurs following freeze–thaw cycles in soil. Freezing and thawing of soil results in death of susceptible microbial populations. For example, Biederbeck and Campbell (1971) found that one freeze–thaw cycle in recently cropped soil resulted in death of 92% of the bacterial population, 55% of the fungi, and 33% of the actinomycetes. The organic substances constituting the cell mass of the deceased organisms provided carbon and energy as well as a variety of cofactors for replication of the surviving microbial populations. This nutrient reservoir plus the reduction in space limitations results in a rapid increase in microbial biomass. Practical implications of freeze–thaw cycles interactions with soil biogeochemical cycles and microbial populations, especially relating to the more frequent and higher intensity freeze–thaw cycles anticipated in high-latitude regions due to anticipated global climate change, have been noted. For example, Haei et al. (2011) examined the impact of freeze–thaw cycles in soils from a Swedish boreal forest on fungal and bacterial populations resulted in

higher fungal growth and reduced bacterial growth ultimately resulting in an increased fungi/bacterial ratio. Others have noted significant impacts of these temperature cycles on soil respiration as well as soil fertility and carbon sequestration (for example, see Jenkins and Adams 2011; Nielsen et al. 2001).

Soil disturbance, natural through activity of soil animals or the result of anthropogenic intervention such as cultivation, may also induce at least a temporary increase in microbial biomass. The disturbance may release occluded organic matter (i.e. substances trapped in soil aggregates) and/or relieve space or free oxygen limitations to growth. Reduction in the quantities of organic matter sequestered in a variety of cultivated soils and associated changes in microbial populations has been demonstration in a variety of agricultural situations. This relationship between soil management and the quality of the soil as assessed by measuring properties of the microbial community (including biomass, diversity, etc.) has long been appreciated. The initial disruption of the soil structure commonly increases the bioavailability of the sequestered soil carbon resulting in microbial growth. With time, due to the changing properties of the residual organic matter and the decreasing quantities resulting from disruption of the aggregate structure, the microbial biomass level declines and microbes more capable of degrading the more resistant soil organic matter components dominate. For example, plowing of a meadow soil results in decreases in native organic matter and an increase in microbial biomass carbon in the top 6 cm of soil by 40–50% (Angers et al. 1992). Related recent studies for reference of this topic include Bailey et al. 2012; Blackwood et al. 2006; Motschenbacher et al. 2014; Six et al. 2000; Smith et al. 2014.

Stationary phase: For many microbial species in soil, the stationary phase is their primary mode of existence. Growth is limited to the degree that new cell production is dependent upon accompanying cell death. For example, although most of the surface area in soil is not occupied by microbial colonies, colonial development is controlled by the space available for expansion in the region of available nutrient and energy resources. Organisms lacking the capacity to migrate to new habitats soon become constrained by the accessible volume of the soil pore in which they are developing. Thus, division of the parent cells depends upon production of space by death and lysis of companion cells. Similar situations may result from the immobilization of a growth-limiting, essential nutrient into microbial biomass. Again, replication is controlled by the release of the limiting nutrient from biomass via cellular death and decomposition.

Decline (death phase): This portion of the bacterial growth curve is essential for the maintenance of soil community homeostasis. Under relatively constant conditions, system survival depends upon maintenance of the *status quo*. This includes a balance in microbial population size. Population densities may increase in response to ecosystem perturbation, but to maintain community stability, the

augmented population must return to its preexisting density. This conclusion is predicated on the assumption that a permanent change or relatively long-lived modification of system dynamics has not occurred. In that case, all microbial population densities are modulated to the new levels optimal for the newly established system parameters. In community with inputs and outputs in equilibrium, population increases are dampened by such mechanisms as death and decay of the newly synthesized cell mass, predation by protozoa or other predators, and subsequent decay of their populations.

Diauxic growth: In laboratory culture, microbes are generally grown in a medium containing a single carbon and energy source plus a variety of cofactors. The growth curve depicted in Figure 4.4 is characteristics of such a situation. Since soil microbes rarely, if ever, encounter chemically pure growth substrates, this growth model must be modified to reflect the more complex carbon and energy supplies existent in soil and the microbial response to them. If the microorganism lacks the genetic capacity to metabolize more than one of the fixed carbon substrates present in its habitat, the presence of multiple organic compounds is of little consequence. This situation is rare. More commonly microbes with the means of catabolizing a variety of carbon sources predominate in a soil.

In a uniform system with no other limitations, microbial population increases reflect catabolite repression kinetics. Catabolite repression, also called the glucose effect, occurs when the presence of one growth substrate represses synthesis of enzymes necessary for the metabolism of alternate compounds. Thus, utilization

Classical Monod Equations for Microbial Growth

1. Equation for Logarithmic Growth:

$$dM/dt = \mu M$$

2. Growth Substrate Impact on Specific Growth Rate

$$\mu = \frac{\mu_{max}\,[S]}{K_s + [S]}$$

3. Growth Yield

$$y = \frac{-d[S]}{dM}$$

Where μ = specific growth rate, M = microbial biomass, t = time, μ_{max} = maximum growth rate, K_s = saturation coefficient, y = growth yield

Figure 4.4 Classical Monod equations describing microbial growth, cell mass yield, and growth substrate interactions.

of a second substrate for growth cannot occur until the initial substance is exhausted or reduced in concentration below growth supportive levels. This results in diauxic growth kinetics (Figure 4.3). The growth pattern reflects successive exhaustion of the available substrates. Once the first nutrient and energy resource is depleted, a lag in growth is observed before the microbes commence to use the second substrate for energy and carbon. During the lag period, the microbes are synthesizing the enzymes needed for mineralization of the second growth-supporting substrate. This transition between energy and nutrient sources can be repeated several times should the microbes be existing in a complex mixture of metabolizable organic compounds.

Demonstration of diauxic growth in the heterogeneous world of soil microbes can be difficult. A preferred carbon and energy source for a particular soil population may become exhausted in one microsite while an adjacent site retains a reasonable supply of the substance. Thus, in a cross-section of a soil community, a variety of microsites can be envisioned to occur wherein many different organic substances are being concurrently metabolized. Studies in pure culture may predict occurrence of diauxic growth kinetics, but the true field data may not reflect its occurrence because of the nonhomogeneity of the field soil. A more specialized growth curve as exemplified by diauxic growth kinetics may be more significant in a soil contaminated with a mixture of organic substance, such as a soil contaminated by an oil spill where sequential mineralization of the substituents could occur.

4.3 Mathematical Representation of Soil Microbial Growth

A variety of complex mathematical models have been developed to describe microbial metabolism in soil ecosystems. These relationships are commonly based on combinations of simple microbial growth relationships derived from assessment of population behavior in laboratory culture. One set of such equations that may be used to elucidate the nuances of microbial growth in soil are the classical Monod equations (Figure 4.5).

Growth rate relationships to microbial biomass: Equation 1 $(dM/\ dt = \mu M)$ depicts the fundamental association of growth rate with total population biomass present. The specific growth rate (u) is not only intrinsic to the microbe itself (i.e. there is a maximal growth rate characteristic of the organism functioning under optimal conditions) but is also controlled by the physical and chemical conditions of the environment in which the organism exists. In soil, the chemical and physical limitations of the habitat result in microbial population replication at a rate far below its maximum capabilities.

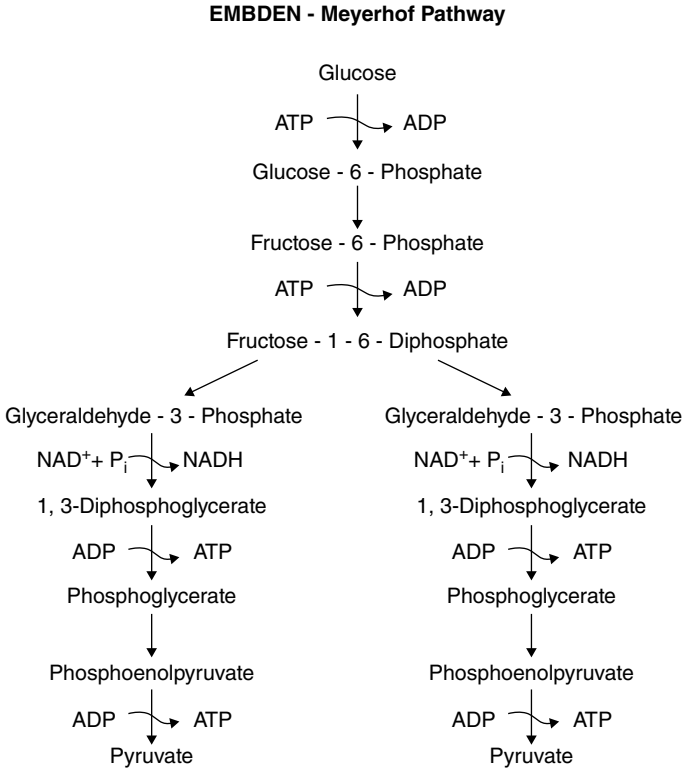

Figure 4.5 Glycolytic conversion of glucose to pyruvate. Note the net production of two ATP and two NADH molecules. Under fermentative conditions, organic intermediates such as acetaldehyde or pyruvate serve as the terminal electron acceptors.

Since bacteria divide by binary fission, the growth rate is related to the generation time as follows:

$$T_d = \text{generation time} = \ln 2 / \mu$$

Under the harsh conditions of the soil environment, generation times of several hours to days are common. These extended generation times compare to division cycles of as short a duration as 10–20 minutes under laboratory culture conditions.

Whereas individual microbial populations in a soil community may be actively replicating, changes in cell mass as well as total microbial biomass may remain unchanged. Individual populations frequently increase or decline, but it is not uncommon to detect a relatively constant level of microbial biomass in an undisturbed soil site. This phenomenon is explained by the fact that (i) most microbes in the surface soil are not replicating – they are in some form of resting state,

(ii) some of the microbial populations are replicating, and (iii) others are declining due to cell death, predation, and so forth. Thus, a small portion of the microbial community could be actively dividing, but their increase in number results in an insignificant alteration of the total soil biomass due to the quasi buffering effect of the inactive and declining portions of the microbial community. Similarly, microbial growth can be shown to be occurring, yet population densities appear to be unchanging. Microbes that decompose fixed carbon in soil are fed upon by a variety of predators and parasites. Thus, the microbes could be consumed by predators at a rate approximating their growth rate. Thus, quantification of growth rate by a method such as ^{3}H incorporation into DNA would indicate an actively multiplying microbial community whereas cell numbers would remain constant.

Growth substrate effects on microbial growth rate: Although it is reasonable to assume that the microbial growth rate in soil is proportional to the concentration of growth-limiting carbon and energy substrates as indicated by Monod equation 2 (Figure 4.4), the relationship is not applicable to soil communities. It is essentially impossible to determine the concentration of growth substrate occurring in the soil microsites where microbial growth occurs. Assumptions underlying the use of concentrations of total substrate in a soil sample as a predictor of microbial growth rate are (i) the substrates and microbes are evenly distributed throughout the soil sample and (ii) all of the carbon and energy sources present in the culture (or vicinity) of the microbial cell are equally available for catabolism. These assumptions clearly are inapplicable in a heterogeneous system such as soil.

Along with the variability in microbial oxidation of their energy sources resulting from the admixture of living cells, soil particulate mineral matter, and colloidal organic matter, heterogeneity of fixed carbon distribution within the soil matrix also results from the exhaustion of nutrients in microsites due to the activity of resident microbial populations. Once the local nutrient supply is depleted due to microbial growth, subsequent growth rates are controlled by the nutrient input rate. Growth kinetics then are described by diffusion equations (static systems) or flow dynamics (where energy resources are contained in water flowing through soil pores – dynamic systems).

Similarly, adequate energy or carbon sources may be available to the microbes in soil microsites but may occur in a form that precludes microbial attack. That is, they may be water insoluble or retained within water-insoluble structures. Decomposition of these materials and hence the growth rate of microbial populations are controlled by the surface area of the particulate material, the rate of breaching of the protective barriers, or even the dissolution rate of the particulate substance.

Surface area-controlled microbial growth is exemplified by the oxidation rate of elemental sulfur particles. Sulfur is minimally soluble in water, but its oxidation to sulfate provides an excellent energy source to a variety of sulfur-oxidizing bacteria. Physical attachment of the bacteria to the sulfur particle is not only required for

oxidation of this substance but is frequently the rate-controlling factor. Early studies by Volger and Umbreit (1941) revealed the necessity for this physical interaction. Similarly, in an examination of the kinetics of oxidation of sulfur by *Thiobacillus ferrooxidans*, Espejo and Romero (1987) noted that only the bacteria attached to the sulfur particles were capable of growth. Therefore, since the elemental sulfur particles are not soluble in water and direct contact of the microbial cells is necessary for its oxidation, it can be concluded that the surface area of the particles controls not only substrate oxidation rate but also resultant microbial growth since sulfur oxidation is providing the microbial energy supply. Thus, in this situation, microbial growth rate is proportional to substrate surface area, not its concentration.

In contrast, Stucki and Alexander (1987) found that dissolution rate, not surface area of the particles, controlled the decomposition of biphenyl by *Moraxella* sp. *Pseudomonas* sp., and *Flavobacterium* sp. Once the water-soluble substrates were depleted, dissolution rate of the hydrocarbon apparently controlled the microbial growth rates. That is, growth was controlled by the rate of diffusion of the minimally water-soluble biphenyl into the water phase.

Many biodecomposable xenobiotic compounds are amended to soils at micro- and nanogram concentrations per gram soil. This is especially true of a variety of herbicides that are effective at low concentrations. Due in part to the common practice of evaluating biodegradation potential in culture media containing at least a thousand fold these concentrations, it has been concluded that microorganisms could not mineralize the minute quantities of organic substrates (i.e. the conclusion was based on the assumption that no growth indicated no interaction with the pesticide). Several laboratory studies have shown that mineralization of nanogram concentrations of a variety of organic substrates in culture (Simkins and Alexander 1984), in sewage (Simkins and Alexander 1984, 1985), and in soil (Scow et al. 1986). In the latter study, soil was amended with phenol or aniline and incubated in biometer flasks at 70% or −0.3 bar soil moisture. The soil moisture level resembled one that would be commonly encountered in a well-drained field soil. The phenol and aniline were mineralized, but not at rates resembling Monod kinetics. A two-compartment model provided the best fit of the data. These data suggest that biological oxidation can occur in soil at concentrations that would not be anticipated to support synthesis of cell mass.

Growth yield as a measure of biomass production in soil: In culture, the quantity of biomass yielded is proportional to the rate of substrate oxidation (Monod equation 3, Figure 4.4). This relationship is applicable to microbial growth under well-defined cultural conditions where biomass production is determined by oxidation of a known energy source, such as occurs in a chemostat. Because of the simplicity of the relationship and the general ability to quantify microbial biomass and growth substances, application of this relationship to soil ecosystems is tempting. Unfortunately, the idealized conditions of the chemostat are rarely, if ever, replicated in soil. Use of this

relationship with soil systems is precluded (i) by the variable contributions of energy resources to biomass production in soil, (ii) by difficulties in attribution of biomass yields with oxidation of a specific growth substance, and (iii) by complexities in linking biomass synthesis and growth substrate oxidation chronologically in complex soil ecosystems. In soil, a variable proportion of the energy yielded from oxidation of the growth substrate by the slowly replicating cells is utilized for cellular maintenance (maintenance energy). Thus, a clear relationship between energy production and biomass yields is not always evident in soil ecosystems. Furthermore, with the number of fixed carbon and other energy sources available in the soil microbe's habitat, at least a portion of the growth assessed in an experiment could result from oxidation of energy sources not quantified or even considered in the experimental design. Over the duration of an experiment, microbes may catalyze a number of different energy sources.

A further complication associated with soil systems relates to the capability to attribute changes in concentration of energy substrates directly with microbial growth yields. The concurrent observation of products of microbial metabolism in soil is not sufficient evidence to assume their linkage in a cause-and-effect relationship. For example, nitrate is commonly detected in soil samples. This compound is the product of energy production by nitrifiers that oxidize nitrite to nitrate. It could be easily assumed that the nitrite oxidizers in a soil sample arose from the production of nitrate associated with them in their habitat, but this nitrate could as easily have entered the soil from external sources (carried with percolating water) or have been produced by previous generations of nitrite oxidizers. Direct linkage of the two variables, nitrifier biomass production and nitrite oxidation, is appropriate only if their augmentation occurs concurrently. Since this prerequisite cannot be generally assumed to be met, application of the third Monod equation to analysis of soil systems should be avoided.

4.4 Uncoupling Energy Production from Microbial Biomass Synthesis

Cometabolism: Organic carbon compounds may also be oxidized in soils in a manner in which energy production, substrate oxidation, and microbial biomass yields are uncoupled. That is, a microbe produces enzymes that catalyze the oxidation of a compound, but no metabolic benefit is provided to the microbe. The energy required to maintain the microbial cell results from the oxidation of a second substrate, which may or may not be chemically related to the cometabolized material. Many aromatic ring-containing compounds are catabolized in soil through this mechanism. For example, catabolism of a variety of polychlorinated

biphenyls (PCBs) constituting the mixture Arochlor was stimulated by the addition of biphenyl to soil samples (Focht and Brunner 1985). In the presence of the biphenyl, between 48% and 49% of the PCBs were converted to carbon dioxide, compared to less than 2% in the absence of the biphenyl. Similar cometabolic responses have been noted for aniline, phenol, and their monochlorinated derivatives (Janke and Ihn 1989); catabolism of trichloroethylene in the presence of methane (Henry and Grbic-Galic 1991); and chloroparaffinic hydrocarbons (Beam and Perry 1973, 1974). Cometabolism is particularly important in the metabolism of halogenated organic compounds (e.g. Boyle 1989; Wackett 1995).

A fortuitous observation from the view of environmental reclamation is the fact that the energy source and the cometabolized substance need not be present in equivalent amounts. Growth-supporting concentrations of an energy-producing substrate can facilitate the catabolism of nanogram concentrations of the cometabolized substrate (e.g. see Schmidt and Alexander 1985; Schmidt et al. 1987; Wang et al. 1984; Wiggins and Alexander 1988). Thus, amendment of soil with metabolizable substrates may encourage decomposition of contaminating organic compounds present in trace quantities.

Maintenance energy: A proportion of the energy derived from oxidation of growth substrates is used for cell maintenance. This is termed *maintenance energy* (the portion of the energy yielded by substrate metabolism that is not used for biosynthetic purposes). Maintenance energy is used to provide for such basic cellular functions as motility and maintenance of internal osmotic pressure. Under energy-limiting conditions, the growth yield of microbial cells is reduced by the amount of energy expended to meet these needs. The slower the generation time of a microbe, the larger the proportion of energy required to maintain cellular integrity. Because of the long generation times of soil microbial populations and the observation that these organisms are generally energy limited, significant reductions in growth yield results from maintenance energy expenditures by these populations.

An example of the impact of maintenance energy expenditures on growth yield of microbes growing at reduced growth rates was provided by Traore et al. (1982). Reduction of the apparent growth rate in chemostat culture from 0.206 to 0.0125 h^{-1} reduced the growth yield of *Desulfovibrio vulgaris* from 6.65 to 2.06 g mol^{-1}. That is, at the lowest growth rate, 68% of the energy was used for cellular maintenance. This phenomenon combined with the low-free energy efficiencies explain how relatively high respiration rates may be detected in soil with a minimal increase in microbial biomass. (Free-energy efficiency equals the free energy captured by the microbe divided by the free energy available in the substrate oxidized for energy. See the section "Chemoautotrophic Existence in Soils" later in this chapter for a comparison of free-energy efficiencies of soil microbes.) The microbes are replicating, but they are doing so rather inefficiently.

4.5 Implications of Microbial Energy and Carbon Transformation Capacities for Soil Biological Processes

Although elucidation of biochemical pathways utilized by soil microbes and the physiological control of the expression of these capacities generally is studied in axenic culture, the real value of microbial metabolic processes resides in their expression by the organisms in their native environment.

The metabolic diversity of the soil microbial community has long been appreciated. In fact, the list of organic substances that can be mineralized by soil microbes is so extensive that it was considered conceptually to be essentially endless. Soil microbes were deemed to be infallible (the microbial infallibility principle) in their capability to rid our soils of unwanted organic compounds. Thus, it was commonly believed in the mid-twentieth century that microbes that are capable of decomposing essentially any organic compound could be expected to occur in soil. This opinion provided the justification for the optimistic consideration of soil as a vast repository for degradation of organic wastes. With the advent of the chemical era and initiation of what could be termed a time of excessive exploitation of soil resources, this concept of microbial infallibility has frequently been transformed into a feeling of extreme microbial fallibility. Many difficulties have arisen because organic compounds, which contain chemical linkages rarely if ever encountered in biologically synthesized compounds, are produced industrially and added to soil systems on a routine basis. These same compounds may have a negative or positive effect on the function of the soil microbial community, but the primary environmental impact of consideration is their extended persistence. Accordingly, the concept of microbial infallibility has to be modified to include the concept of "biological" synthesis; that is, the soil microbial community is considered to be capable of decomposing all biologically synthesized organic compounds (or synthetic chemicals that mimic the covalent linkages produced biologically), assuming that all other physical and chemical requirements for microbial function are met. It must be noted that this inclusion of a biological synthesis similarity consideration to assumptions regarding environmental longevity of xenobiotic chemicals in soil does not provide insights into their decomposition rate.

4.5.1 Energy Acquisition in Soil Ecosystems

Soil microbes, as do all living entities, derive the energy necessary for cellular function through oxidation reactions. A basic principle of biochemistry is that all oxidation must be balanced by reductions; that is, electrons generated in the oxidation processes must be transported to an electron acceptor. Common terminal

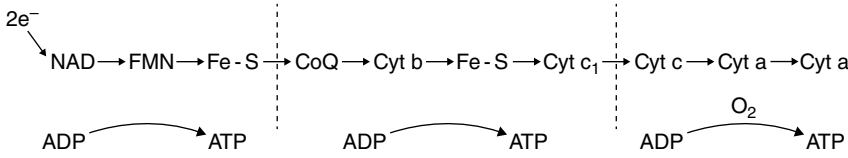

Figure 4.6 Production of ATP via transport of electrons through an electron transport chain with molecular oxygen as the terminal electron acceptor.

electron acceptors include oxygen, nitrate, nitrite, sulfate, and a variety of organic compounds, including acetate and pyruvate.

The quantity of energy provided to the growing cell in the form of adenosine triphosphate (ATP) is dependent upon the intermediate steps between the initial oxidative reaction and the final electron acceptor. A direct transfer of the electron from the oxidized substrate to an organic recipient as occurs in glycolysis (Figure 4.6) yields a single ATP molecule per electron transfer, whereas passage of the electron through a series of intermediate reduction and oxidation reactions (Figure 4.7), as occurs in respiratory metabolism of glucose through the citric acid cycle, yields as many as three ATP molecules per electron transferred. Thus, the incomplete oxidation of glucose to organic intermediates by glycolysis produces a net gain of two ATP molecules, whereas a maximum of 38 ATP molecules is produced by the complete conversion of glucose to carbon dioxide (Figure 4.8).

Aerobic versus anoxic processes: Environmental metabolic processes can conveniently be grouped into two rather broad categories: aerobic and anoxic conversions. Maximal biological energy is made available for cellular function when free oxygen is the terminal electron acceptor. Inversely, incomplete oxidation of fixed carbon substrates in the absence of free oxygen results in reduced energy recovery and reduced biomass production. Based on the above discussion of substrate-level phosphorylation and the recovery of energy through an electron-transport chain, such conclusions are reasonable, and are applicable to the majority of metabolic processes.

Fortunately, the underlying assumption of this generalization – cytochrome-based respiration (the basic process when molecular oxygen serves as the terminal electron acceptor) only occurs under aerobic conditions – is *not* true. Denitrification (e.g. Koike and Hattori 1975; Kristjansson et al. 1978) and sulfate reduction (e.g. see Kim and Akagi 1985; Postgate 1984), two conversions that require anoxic (oxygen free) conditions, yield energy to the growing cell through cytochrome-based electron-transfer chains similar to that depicted in Figure 4.5. Thus, for these two anoxic processes, the quantity of cell mass produced approaches that achieved when free oxygen is the terminal acceptor.

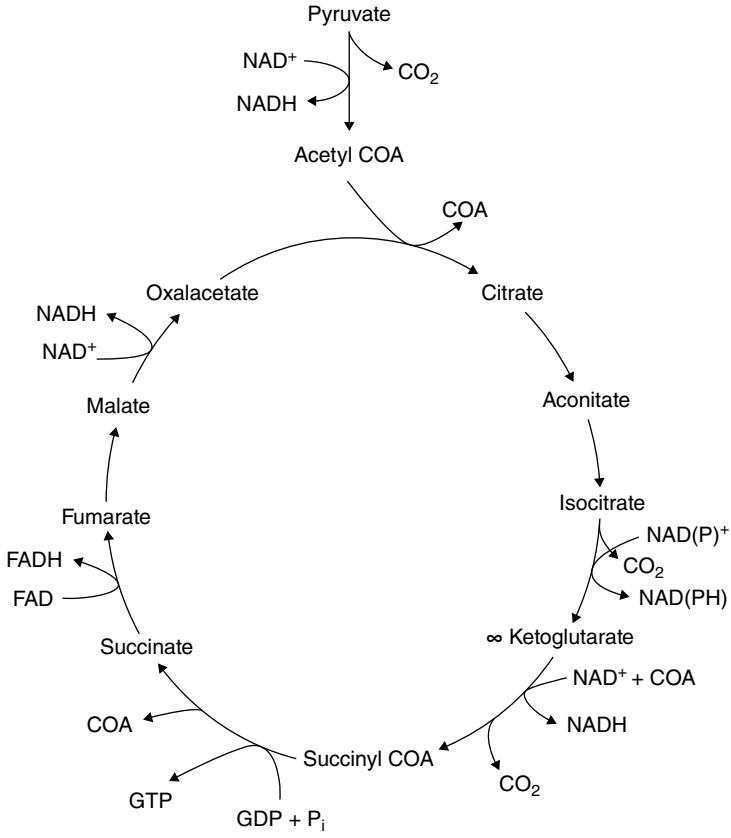

Figure 4.7 The tricarboxylic acid cycle (TCA).

A basic principle to be considered in these dissimilatory processes (oxidation of carbon substrates coupled to cytochrome oxidation under anoxic conditions) is that the utilization of the specific electron acceptors is mutually exclusive. That is, the dissimilatory reduction of nitrate or sulfate does not occur until the molecular oxygen supply in the microsite of the microbial cell is exhausted. This results in a reduction in the local reduction–oxidation potential sufficient to allow utilization of the alternate electron acceptors. Each of these substances functions at specific reduction–oxidation potentials (Table 4.1). That is, the redox potential of the environment of the microbial cells is perched at the specific value associated with a given electron acceptor until it is exhausted, then the potential drops to a new level characteristic of the next electron acceptor. For example, in a system containing nitrogen oxides (but no molecular oxygen), the reduction potential remains at the level dictated by the nitrogen oxides until all of them have been reduced through denitrification, then organic substances or other acceptors

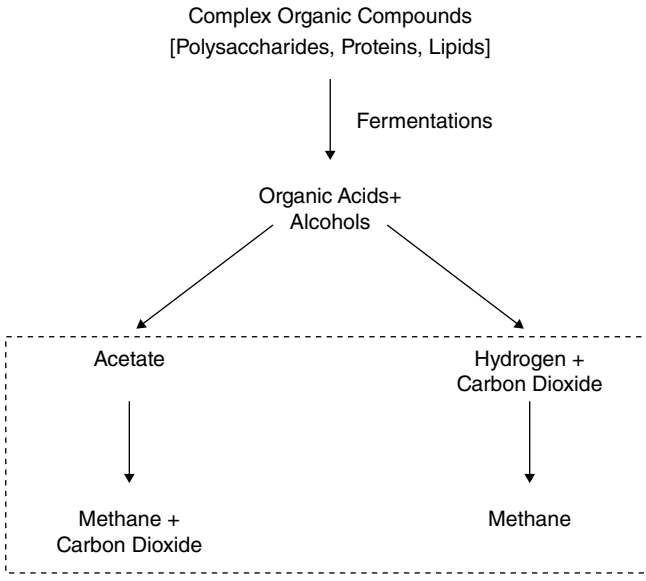

Figure 4.8 General organic matter transformations associated with methanogenesis. Processes enclosed in the box are catalyzed by methanogenic bacteria.

(e.g. sulfate), if present, become the primary terminal electron acceptors for the active biological community.

A complex example of this phenomenon involves the sequential oxidation and reduction of growth substrates and terminal electron acceptors in a flooded soil containing nitrate, ammonium, manganese (II), and ferrous iron (Patrick and Jugsujinda 1992). No overlap in the oxidation or reduction of nitrate and manganese was detected, and little overlap in the transformation of manganese and ferrous ions occurred.

The environmental implications of the potential for dissimilatory oxidation of fixed carbon with nitrate or sulfate as the final electron acceptor include the fact that greater biomass is supported by the processes than occurs with classical fermentation reactions, and that the organic substances providing the energy are completely oxidized to carbon dioxide and water. The latter trait is particularly significant in bioremediation processes (Tiedje et al. 1984). This is especially important in situations where the oxygen supply is exhausted due to the rapid catabolism of the energy-supplying substrate. Should nitrogen oxides or sulfate be present, the contaminant could still be reduced rapidly to carbon dioxide and water even under the anoxic conditions.

The potential for exploiting dissimilatory processes under anoxic conditions for decomposition of soil contaminants is underscored by the variety of substrates catabolized by denitrifiers and sulfate reducers. Denitrifiers have been shown to

Table 4.1 Reduction potentials of some common electron acceptors functioning in soil biological systems

Redox couple	E_0 (volts)
O_2/H_2O	0.82
Fe^{3+}/Fe^{2+}	0.77
NO_3^-/NO_2^-	0.42
Cytochrome c oxidized/reduced	0.25
Acetaldehyde/ethanol	0.20
Cytochrome b oxidized/reduced	0.07
SO_4^{2-}/S^{2-}	−0.22
NAD^+/NADH	−0.32
CO_2/CH_4	−0.35
H^+/H_2	−0.42

oxidize a variety of aromatic ring-containing compounds, including phthalic acid (Aftring et al. 1981; Nozawa and Maruyama 1988), toluene (Altenschmidt and Fuchs 1991; Evans et al. 1991a), xylene (Evans et al. 1991b), benzene and a variety of alkyl benzenes (Hutchins 1991), resorcyclic acids and resorcinol (Kludge et al. 1990), plus the more complex polycyclic aromatic hydrocarbons (McNally et al. 1998; Mihelcic and Luthy 1988a, b). An added advantage of oxidation of these substances via denitrification is that a second soil pollutant, nitrate, is also removed from the system through its conversion to nitrous oxide and dinitrogen. Similarly, sulfate can serve as a terminal electron acceptor for oxidation of such compounds as chlorophenol (Haggblom and Young 1990), aniline and dihydroxybenzenes (Schnell et al. 1989), benzoate (Tsaki et al. 1991), catechol (Szewzk and Pfennig 1987), and m-cresol (Ramanand and Suflita 1991). Microbial decomposition of similar aromatic compounds with sulfate or nitrate as the terminal electron acceptor by microbial consortia in anoxic conditions is exemplified by Li et al. (2012) and So et al. (2003).

4.5.2 Microbial Contribution to Soil Energy and Carbon Transformation

The diversity of habitats available to soil microbes is reflected in their versatility in carbon and energy metabolic capacities. Essentially all microbial groupings (Table 4.2) are found at least to a limited extent in soil ecosystems. Some are important contributors to total microbial biomass and are versatile in their capacity to oxidize fixed carbon substrates (e.g. the heterotrophs), whereas others,

Table 4.2 Some groupings of soil microbes based on their primary energy sources

Microbial group	Energy source	Carbon source	Terminal electron acceptors	Examples
Heterotrophs				
Aerobes	Fixed carbon	Fixed carbon	O_2, NO_x, SO_4^{2-}	*Arthrobacter* spp. *Pseudomonas* spp. *Bacillus* spp. Denitrifiers
Anaerobes	Fixed carbon	Fixed carbon	Fixed carbon	*Clostridium* spp.
Fermenters	Fixed carbon	Fixed carbon or CO_2	Fixed carbon	Enterics, lactic acid bacteria
Autotrophs				
Photoautotrophs	Light	CO_2	CO_2	Methanogens algae, photosynthetic bacteria

although limited contributors to total soil microbial biomass, are important because of their pivotal role in soil biogeochemical processes (e.g. nitrifiers). Others pique the interest of soil microbiologists not because of the impact of their presence on soil system function, but more due to their unusual or precarious existence in the system (e.g. soil algae or photosynthetic bacteria). Whether they are major or minor components of the total soil microbial biomass, all replicating microbial are important, if not essential, to total soil ecosystem function.

Chemoautotrophic existence in soils: Chemoautotrophs gain their energy through the oxidation of inorganic compounds. Primary electron sources are ammonium, nitrite, hydrogen, and a variety of sulfur-based anions. A characteristic of these microbes is their specificity for particular energy sources. For example, autotrophs oxidizing nitrogenous compounds use only ammonium or nitrite as electron sources, whereas sulfur autotrophs are restricted to oxidation of sulfur compounds. Furthermore, the specificity generally extends to individual compounds within each grouping – for example, nitrifiers are divided into ammonium and nitrite oxidizers. Chemoautotrophs may assimilate simple organic compounds, but their primary cellular structure is provided through the reduction of carbon dioxide. Many of the autotrophic bacteria are obligate autotrophs; that is, they can use only carbon dioxide as a carbon source. Facultative autotrophs are capable of growing either autotrophically or heterotrophically.

Chemoautotrophic organisms are of pivotal importance to soil biotic function. Although their contribution of fixed carbon to the ecosystem is minor compared

to that incorporated by higher plants, oxidation of their inorganic substrates is frequently central to the completion of various soil biogeochemical cycles. For example, ammonium oxidation to nitrate is primarily catalyzed by autotrophic nitrifiers in soil.

Although neither heterotrophs or chemoautotrophs capture all of the energy released from oxidation of their substrate, chemoautotrophic bacteria are especially inefficient in energy recovery (free energy efficiency). The free energy efficiency is generally measured in culture by quantifying the heat of combustion of the cells produced by metabolism of a specific substrate and the heat of combustion of the quantity of substrate oxidized in this process. This is not measured directly in soil because of the difficulty in determining the specific substrate catabolized among the vast array available to the cell. The free energy efficiency can be as low as 5–10% for oxidation of nitrite to nitrate by *Nitrobacter* species (Alexander 1977) to 50% for the oxidation of elemental sulfur to sulfate by *Thiobacillus thiooxidans*.

For the obligate autotrophs, all of the cellular substituents must be synthesized from the reduction of carbon dioxide. Considering that the conversion of ammonium to nitrate yields 66 kcal of energy per mole of substrate oxidized, and the oxidation of nitrate to nitrate produces only 20 kcal of energy per mole of substrate, and that the cellular substituents of both the ammonium and nitrite oxidizers are the same, approximately threefold more nitrite must be oxidized to produce the same biomass of nitrite oxidizers as for the ammonium oxidizers. Approximately 35 nitrogen atoms are oxidized per carbon fixed for the ammonium oxidizers as compared to about 100 nitrogen atoms for the nitrite oxidizers. Thus, the microorganisms using these low-energy substrates must oxidize large quantities of their substrates to produce the same biomass as those growing by more efficient metabolic processes.

In summary, chemoautotrophs are a small portion of the total soil microbial biomass but they are essential participants in the provision of soil ecosystem services. A major role in ecosystem function and sustainability resides in their capacity to oxidize and to reduce soil mineral components. For example, they convert ammonium to plant available nitrate, oxidize a variety of reduced sulfur compounds to sulfate, and oxidize ferrous iron to ferric iron. Conversely, examples of reductive autotrophic processes include conversion of nitrate to dinitrogen, thereby closing the loop of the nitrogen cycle and reduce sulfate to elemental sulfur and sulfide.

Anaerobic heterotrophs and the soil ecosystem: Anaerobes are microorganisms that grow and reproduce in the absence of free oxygen. (Organisms using cytochrome-based electron-transport systems, e.g. sulfate reducers and denitrifiers, even when functioning under anoxic conditions, are by definition excluded from this class of organisms.) Although there is in reality a gradient of sensitivity to free oxygen, oxygen is lethal to anaerobes.

Anaerobic microbes may be divided into two classes, strict or obligate anaerobes, and facultative anaerobes. Strict anaerobes do not grow or survive in the vegetative phase for extended periods in the presence of molecular oxygen. Examples of this group of organisms include the clostridia (which are ubiquitous in soil), methanogens, protozoa, and anaerobic fungi. Facultative anaerobes are capable of growth in the presence or absence of free oxygen. Examples of these organisms include the commonly encountered enteric bacteria.

In spite of their sensitivity to molecular oxygen and the abundance of this molecule in our terrestrial system, anaerobic microbes are nearly ubiquitous in soil systems. In fact, in a well-aggregated soil, anoxic and aerobic processes generally occur concurrently, as the result of the intermixing of aerobic and anoxic sites in and around the individual soil aggregates. For this to occur, aerobic bacteria and fungi consume molecular oxygen on or near the surface of a soil aggregate at a rate sufficient to lower the free oxygen levels sufficiently that essentially no free oxygen remains to diffuse into the aggregate. Thus, the internal portions of the aggregate become anoxic. Since it is not uncommon for the nutrients requisite to support growth of anaerobic microbes to be contained within the aggregate structure, these populations not only survive but contribute significantly to overall soil metabolic activity.

Flooding of the soil, as can result from a heavy rainfall, may result in expansion of the anaerobic microbial biomass. It must be stressed that a flooded soil is not necessarily an anaerobic soil. The oxygen tension of a flooded soil system depends on (i) the oxygen diffusion rate in water, (ii) the rate of consumption of this molecule by the living community plus any depletion due to chemical processes, as well as (iii) the potential for oxygenation of the sites through influxes of oxygen-bearing water. That is, a flooded system in which the water level is maintained by inputs of oxygenated waters may not become anoxic. Soil sites commonly associated with oxygen-depleted conditions are swamps or marshes, rice paddies, sediments, and any other sites receiving inputs of easily decomposed organic matter. The latter soils include a variety of environmentally stressed situations such as buried organic matter in landfills or soils affected by spills of biodecomposable societal products.

The products of complete mineralization of an organic substance containing carbon, hydrogen, oxygen, and sulfur in the absence of oxygen are carbon dioxide, methane, ammonium ion, hydrogen sulfide, and water. This list of products contrasts to the carbon dioxide, ammonium ion, water, and hydrogen sulfide yielded by aerobic decomposition of the same compound. Of more general importance to soil systems are the products of incomplete mineralization of organic substances by facultative and obligate anaerobic organisms. In these situations, alcohols and organic acids accumulate. These fermentations leave a major portion of the energy contained in the substrates in the fermentation products. Thus, microbial biomass production is limited compared to what would be produced through complete, aerobic oxidation of the fixed carbon sources.

Methanogenesis: Methane generation provides an excellent example of complete mineralization of organic compounds under anaerobic conditions and the interaction of a variety of anaerobic bacteria to achieve these ends. Complex substrates are decomposed by anaerobic and fermentative bacteria to the simple substrates that can be converted to methane. Methane synthesis is continuous in marshes and bogs where the extremely reducing environment necessary for the process (−200 to −1000 mV) commonly occurs.

Methane is produced by a highly specialized group of obligately anaerobic microbes, the methanogenic bacteria. This unique group of anaerobic bacteria includes the following species: *Methanobacterium arbophilicum*, *Methanobacterium formicum*, *Methanobacterium rumination*, *Methanobacterium mobile*, *Methanobacterium thermoautotrophicum*, *Methanococcus vanielli*, *Methanosarcina barkeri*, and *Methanospirillum hungatii*. These organisms reduce carbon dioxide to methane with hydrogen generally serving as the electron donor by the following reaction:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

Therefore, hydrogen is serving as a sole source of reducing power for both methanogenesis and cell carbon synthesis. Other substrates that can be converted to methane include methanol, formate, acetate, and methylamines. With the latter substrates, the carbon substrate serves as both the electron donor and the final electron acceptor. For example, acetate is converted to methane and carbon dioxide as follows:

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$$

The acetate, carbon dioxide, and hydrogen used by these methanogenic bacteria are generated by the fermentation of complex polymers, including cellulose and other polysaccharides, by obligate anaerobes, and fermentative bacteria (Figure 4.9). When it is considered that most other carbon pools entering soil are products of decomposition of plant based organic carbon compounds (animal biomass, composts, and sludges, as well as petroleum products), the intense dependence of belowground life processes on plant productivity is highlighted.

Transformation of long-term stable soil organic matter pools: Biomass carbon entering soil can be divided into two primary pools: biodegradation resistant and readily or easily metabolized carbon. Clearly, the biodegradation-resistant categorization is a relative term in that these substances (e.g. lignified materials) are decomposed in soil, albeit slowly. The readily metabolized fractions are defined as those fixed carbon materials that provide a carbon and energy source for the soil microbial community with minimal energy expenditure by the microbe. Included in these

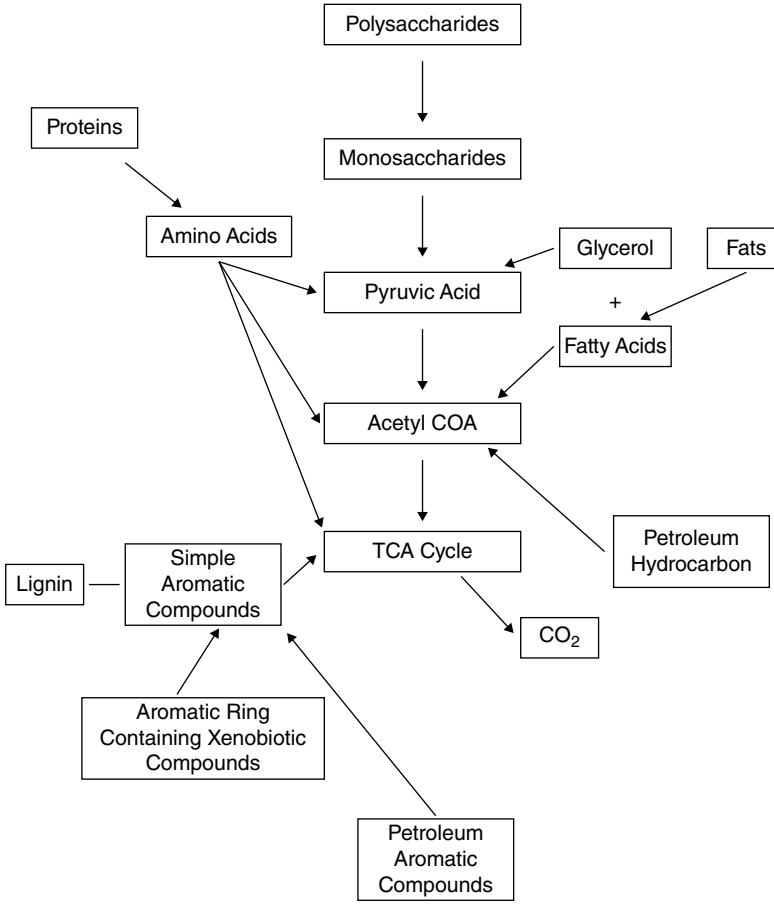

Figure 4.9 Conceptual model of carbon catabolism by the soil heterotrophic microbial community.

energy expenditures are the activities necessary to synthesize catabolic enzymes as well as those necessary to activate the substrate molecule to enhance its degradation susceptibility – for example, the phosphorylation of hexoses with ATP to form hexose-monophosphates (Figure 4.5). Lignin and humic acid do contain sufficient chemical energy to support growing microbes, but the energy expended to synthesize the array of enzymes required for the process far exceeds the amount of energy liberated by the total mineralized of the complex aromatic ring structures.

Occurrence of necessary but undetectable metabolic activities: It is not unusual to know *a priori* that a certain metabolic activity must be present in a soil

sample, but due to difficulties associated with analysis of a complex mixture of abiotic and biotic components, detection, or quantification of the process is precluded. Fortunately, the properties of comparative biochemistry dictate that metabolic reactions occurring in bacteria or fungi that can be studied in laboratory culture are common with "minor variations" to those occurring in the soil microbial community. Thus, if a fixed carbon substance is amended to a soil sample and its decomposition is documented, it is reasonable to conclude that the decomposition is occurring, with minor variations, by commonly described biochemical pathways – for example, Embden Meyerhoff Parnas, citric acid cycle pathways.

This observation allows construction of a model predictive of the fate of the primary classes of organic carbon compounds entering soil (Figure 4.9). As in other ecosystems, in soil, most fixed carbon is degraded to simple organic acids that can be activated to acetyl-coA and fed into the tricarboxylic acid cycle. For example, proteins are hydrolyzed by proteases and deaminated to organic acids. Aromatic ring-containing substances are hydroxylated to facilitate ring cleavage (Figure 4.10). In this process, molecular oxygen is incorporated directly into the aromatic ring. Thus, it is logical to predict that within any soil sample containing viable microbial populations, all of the common enzymes for intermediary metabolism associated with these cycles plus those activities, such as proteases and hydrolases necessary to produce the precursors of these cycles, are present in that soil sample.

Humification: The complexity of the soil system, including the potential for a variety of spontaneous chemical reactions, provides a number of fates for reactive biochemical intermediates that may not be detected in laboratory culture. Essentially any reactive organic compound entering soil could be incorporated into soil humic acids; that is, it can be humified. Since many of the reactions associated with covalent linkage of organic compounds to humic acids are chemically catalyzed, the reaction rate is controlled in large part by the reactivity of the substrates and the probability of interaction of the reactants. In typical soils containing less than 2% organic matter, the probability of such reactions is limited, although significant. Since in most soil systems the factor controlling the rate of humification is the probability of collision of the reactants, the survival time of the molecule in soil and its concentration in soil become major predictor of whether the substance will be decomposed totally or at least partially, or whether it will be humified. The complex humic acid molecules are formed through a combination of biologically catalyzed reactions and spontaneous chemical reactions. The more long-lasting organic components, such as complex polyaromatics in petroleum or lignin, are more likely to be humified than the more ephemeral proteins and simple sugars.

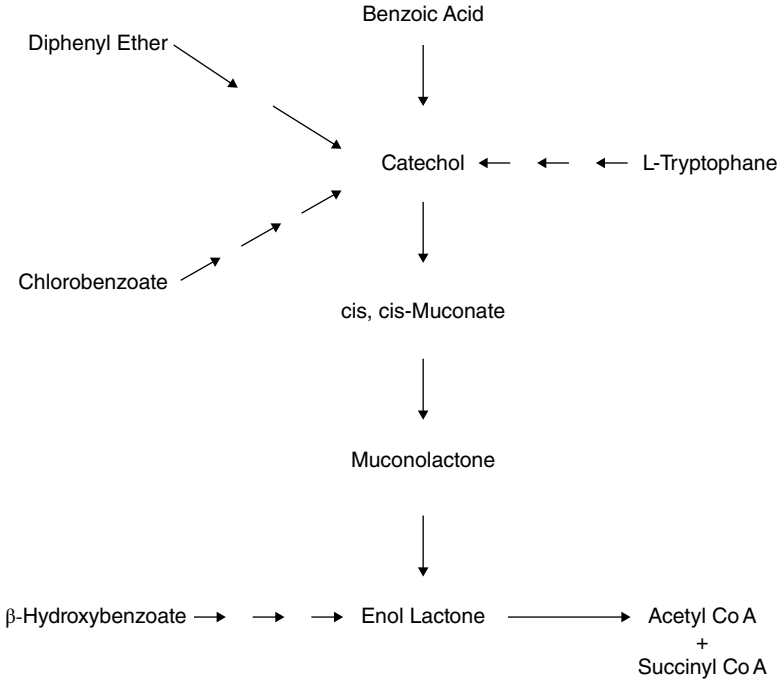

Figure 4.10 Examples of pathways leading to entry of the carbons of aromatic compounds into the TCA cycle. The specific pathway depicted is the catechol pathway. Other aromatic ring-degrading processes include the gentisate and protocatechuate pathways.

4.6 Concluding Comments

The soil microbial community is clearly distinguished by its diversity in metabolic capacity. Along with the common variety of fixed carbon substances decomposed by living organisms, soil microbes also oxidize a number of soil minerals (e.g. sulfides), metals (e.g. ferrous ion), and several cations and anions containing atoms existing in numerous oxidation states (e.g. ammonium and thiosulfate). Combined with the capacity to utilize several different terminal electron acceptors (e.g. molecular oxygen, nitrate, nitrite, sulfate, and numerous organic compounds), microbial life is capable of existing in soil under essentially any chemical and physical condition that allows for sustenance of cellular integrity. The societal legacy of these myriad metabolic capabilities is the potential for the soil microbial community to survive severe chemical insults and to return to previously existing conditions. For example, influxes of acidic solutions of sulfate (acid mine

drainage) may be ameliorated by anaerobic reduction of the sulfate to sulfide (i.e. the sulfate is used as a terminal electron acceptor). Toxic organic compounds may be mineralized in the presence or absence of molecular oxygen. Heavy metal contaminants can be removed from interstitial waters by precipitation with the sulfide produced by sulfate reduction. Survivability and sustenance of the soil microbial community are built to a large extent on its vast catalog of catabolic capabilities.

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5

Process Control in Soil

Accurate prediction of the response of soil biological communities to environmental perturbation is predicated upon the premise that an accurate or at least a reasonably representative understanding of the ecosystem properties controlling soil processes has been developed. This prerequisite presents a meaningful conceptual problem to those seeking to understanding basic soil microbial processes. Microbiological principles and metabolic reactions are understood at the level of the individual microbial cell, colony or the molecular level (i.e. the microlevel), whereas soil properties delimiting the expression of microbial potential are assessed at a macrolevel (e.g. ranging from several grams of soil to a total ecosystem characterization). Thus, development of a true picture of the effect of soil microbes on large-scale ecosystem function requires extrapolation of the microsite and macrosite processes. These three spheres of concern (microsite, ecosystem-wide, worldwide) are not always easily merged, but such reconciliation of data is essential. The task would be simpler were microbial function controlled by one or a few physical, chemical or biological soil properties. In reality, microbes in soil are responding to a combination of soil properties acting in concert. For example, it is relatively easy to dismiss a proposed 1–3 °C rise in soil temperature due to climate change as being insignificant. Yet, soil temperature variation due to climate change has a large effect on the hydrological cycle (occurrence of droughts or floods as well as the seasonal variation of soil water content) at a local level. Additionally, the anticipated declines in soil water result in increased soil salinity, osmotic and matric potential impacts on microbial activity (e.g. Asghar et al. 2012; Chowdhury et al. 2011). Clearly, the soil microbe is encountering a complex, stressful environment.

Macro- and microsite reality and ecosystem heterogeneity: A primary difficulty associated with the merging of microbial and soil chemical and physical analyses results from the high degree of variability of soil properties both in a vertical and horizontal plane within the soil profile. A false impression of the microsite traits may be gained from study of the relatively large soil samples, both *in situ* at the soil site as well as in

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soil samples incubated in the laboratory or greenhouse. Values for soil properties, such as pH or cation exchange capacity, are meaningfully variable at microdistances within a soil. Soil properties measured in a sample of soil as small as $10\,cm^3$, for example, may be quite different from those measured in a large soil sample.

This telescoping nature of the application of soil microbiological research reveals a characteristic and, to some degree, an intellectual trap of our discipline. Data are necessarily a summation of a variety of reactions occurring in highly variable soil microsites. Thus, a composite representation of soil processes must result in the emphasis of dominant reactions, but other processes of major importance to ecosystem integrity and function may be overlooked or the importance of their contribution to system complexity minimized. For example, the bulk of terrestrial soils are aerobic. Molecular oxygen diffuses freely into the pores of nonwaterlogged soils as well as being transferred deep into the soil matrix through mass transfer driven by water infiltration. For example, mass transfer of atmospheric gases into soil can occur when groundwater is drained from the soil or as a result of the percolation of oxygen-bearing irrigation or rainwater through soil pores.

Thus, it is easy to emphasize aerobic processes in conceptual models of ecosystem function to the detriment of anoxic reactions. Yet, we know that anaerobic soil bacteria occur and are active in essentially all soils and that the products of anaerobic microbial activity are essential for ecosystem survival (e.g. denitrification). Our understanding of soil microbial processes is constructed on a seemingly contradictory concept of the system. The occurrence of both aerobic and anoxic microbial processes in close proximity in most surface soils provides an excellent example of this quandary. Obligate aerobic processes, such as aerobic nitrification (oxidation of ammonium to nitrate), occur in close physical proximity in soil to obligatorily anoxic processes, such as denitrification (reduction of the nitrate produced by nitrification to nitrous oxide and dinitrogen). The distribution of aerobic and anaerobic microsites in close proximity in surface soils and the diffusion of nitrate into the anoxic sites following its synthesis by the nitrifiers allows this seemingly contradictory interlocking of these incongruent reactions to occur.

Thus, a foundational concept for this analysis of process control in soil is that all microbial processes are first the summation of the activity of a variety of individuals, whether a cell, multicellular complex (that is, a biofilm), or even a collection of enzymes. Second, the level of activity of the microbe is controlled by the summation of a complex, interacting soil physical and chemical properties. Initial considerations involve an evaluation of the concept of a limiting factor and experimental quantification of the impact of various environmental properties on biological activities. This will be followed by elucidation of the variation of specific soil chemical and physical traits. Soil properties interacting with biological entities to be evaluated herein include nutrient requirements, soil moisture, oxygen tension, redox potential, pH, and temperature.

5.1 Microbial Response to Abiotic Limitations: General Considerations

5.1.1 Definition of Limitations to Biological Activity

The classical definition of an environmental factor that determines the rate of a biological activity can be summarized by Liebig's law of the minimum: "Under steady-state conditions, the essential material available in amounts most closely approaching the critical minimum needed by a given organisms will tend to be the limiting one" (Dommergues et al. 1978). This principle was originally developed to describe the impact of variation of nutrients on microbial growth and activity, but it has been expanded in recent years to include a number of growth limitors, including temperature and a variety of inorganic and organic metabolic inhibitors.

It must be stressed that the application of Liebig's law to a particular ecosystem is appropriate only if the system is at steady state. This condition is necessitated by the fact that the organisms and their interactions cannot be adequately assessed in relationship to rate-controlling soil conditions except at steady state; otherwise, populations and/or their activities may be increasing or decreasing to reach the density or activity level allowed by the newly established environmental conditions.

Although it is conceptually attractive, Liebig's law is of limited applicability for describing the dynamics of soil ecosystems. This results from three conditions.

- As exemplified above in regard to the complexity of changes in soil properties due to drying, soil microbes in general must deal with an array of stress factors, only in extreme situations, such as highly acidic or temperature stressed situations would the microbes have the luxury of dealing with a single stress factor.
- This combined stress produces different responses by the microbial community than would be anticipated to result from any soil property acting singly on the microbial cells.
- The structure of the soil ecosystem makes it difficult to determine the exact conditions at the microsite where the organisms are living.

5.1.1.1 Microbial Responses to a Stressful World

Limitations to microbial growth are generally due to a combination of biological, chemical and physical soil properties acting in concert. It is rare for one soil property to preclude life processes. Exceptions to the multiplicity of stresses may be exemplified, to some degree, by consideration of microbial populations in the dry deserts of Antarctica (Benoit and Hall 1970; Cameron 1972) or incidence of extreme anthropogenic intervention (e.g. acidification due to acid mine drainage, nuclear power plant cooling water outflows, or soils receiving massive influxes of heavy metal-containing mine wastes). Essentially, all soil microbes are subject to

compromises in the rates at which they function. More commonly, a combination of several soil physical and chemical conditions stresses soil microbial populations sufficiently to cause them to operate at suboptimal levels. The effect of interacting soil properties on microbial processes frequently cannot be predicted by examining microbial behavior in laboratory culture. It may be predicted from laboratory studies that a microbial population of interest would be unaffected by a particular soil property; yet, in native soils, the growth of the organisms is precluded under seemingly acceptable conditions or its range of occurrence is truncated. For example, fungi grow in culture within the pH range of 3.0–8.0, but in soil the practical range is closer to 3.0–6.5. In this case, the impact of a chemical controller is altered by the microbe's capacity to compete with cohabiting biological populations. That is, the activity of soil fungi is controlled by soil pH plus the organisms' ability to compete with soil bacteria. The latter populations are better competitors at the higher pH than are the fungi. Similarly, some environmental properties may compensate for cellular inadequacies, thereby allowing microbial populations to develop outside the range predicted from laboratory studies for its maximum, minimum, or optimal activity. For example, it is not unusual to isolate soil microbes capable of growth at 30 °C in culture but not at 37 °C in a particular medium. If growth factors (or vitamins) are added to the culture medium, the organisms grow quite well at the higher temperature. This results from the fact that an enzyme essential for the production of the growth factor by the microbes could be temperature sensitive; that is, it is inactive at the higher temperature. Availability of this obligatory growth factor in the environment allows the previously inactive organism to proliferate in the seemingly detrimental habitat. Therefore, if a microbe is growing in the presence of an organism producing the compensating growth factor (e.g. another microbe or evan a plant root), its temperature tolerance will be extended.

A further difficulty with application of Liebig's law of the minimum to conditions in native soil systems is more of a practical concern than a conceptual difficulty. Due to soil spatial and temporal variability, data may imply nonexistent restrictions to microbial growth. Basically, these observations result from the fact that a microorganism must physically encounter an inhibitory or stimulatory soil component before its growth or activity is affected. Microbes generally grow as microcolonies in soil microsites where their needs are met. Thus, microbial growth is limited by the distribution of carbon, nitrogen, and other nutrients; aeration; anoxic conditions; and so forth. Any troublesome soil property occurring within a short distance from the perimeter of the colony is nonexistent from the viewpoint of the microbes. Furthermore, environmental conditions vary on a daily, seasonal, and annual basis. This is easily envisioned by consideration of the rise in temperature in the surface centimeter of soil on an early spring day. In the early morning hours, the temperature may be suboptimal for microbial activity;

perhaps the soil is frozen. As the hours pass, soil temperature increases, resulting in a stimulation of biological processes until some optimal or maximal rate is reached. On the rare hot days, temperature combined in association with the resultant increased desiccation rate (soil moisture loss) could present a situation that is inhibitory to the life processes occurring in the very top portion of the soil profile. Similar temporal effects could be described for other variables, such as nutrient concentration, pH, and moisture. Nutrients may be leached through soil during rainfall events that clearly cause dramatic changes in soil moisture levels. Similarly pH variation may occur at the microsite level due to acid production by the microbes themselves or result from a periodic influx of human products, such as acid rain or acid mine drainage waters. Thus, analysis of the soil site at a particular time of day or season of the year may indicate that the growth-promoting or -inhibiting conditions do not exist, whereas if the measurements were taken a few hours earlier or later, contrasting results would be obtained.

These observations indicate the necessity of modification of our concept of a limiting factor to fit the complexities of soil. A more appropriate basis for consideration of control of soil microbial process is the following: "The growth of a soil microbiological population depends on a combination of several limiting factors acting collectively and interdependently at the soil microsite wherein the microbe is functioning." For example, denitrifying bacteria in soil can be limited by variation in easily metabolizable carbon levels, nitrate concentrations, as well as oxygen concentrations. Autotrophic nitrifiers are frequently controlled by soil pH, ammonium concentrations, and oxygen tension. It is likely reasonable to assume that none of the soil properties at the microsite where the microbes grow are optimal for the organism. Thus, it can be concluded that the specific level of activity of each individual is controlled by the combined effect of nearly all physical, chemical, and biological properties of the site. Again, exceptions would be those times or situations when one soil property occurs at such an extreme value that its effect overrides all others (e.g. soil moisture so low that minimal cellular respiration is possible, or temperatures approaching the maximum tolerated level). In the majority of the situations, though, it is the impact of the combination of limitations acting in concert that determines the extent of microbial population development in a soil site.

5.1.2 Elucidation of Limiting Factors in Soil

Under most circumstances, the complexities of the soil environment as well as the microscopic and submicroscopic size of the soil microbial community preclude clear determination of limiting factors in the field. In some situations, field observations are quite useful (e.g. Antarctic dry deserts or anthropogenically affected sites where extremes in physical or chemical conditions are easily discerned), but

for the vast majority of the terrestrial ecosystem, controlled, laboratory-based study of the system is required to quantify the contribution of each individual soil property to biological reaction rates. Options for determining biological activities in soil include variation of single or combinations of properties in laboratory-incubated soil samples or assessment of field activities in several diverse systems.

5.1.2.1 Limitations of Laboratory-Generated Data

Scientific conditioning creates a desire of experimenters to study the effect of variation of each individual soil property on biological processes under controlled conditions in the laboratory. This offers the security of simplicity and reproducibility. In laboratory experiments, soil conditions may be varied singly or in concert, and the resultant impact on microbial activity assessed accurately. Easily manipulated factors can include pH, temperature, moisture, and nutrient levels. These conceptually alluring studies may yield results that describe the capacity of existing microbial populations in the soil system to cope with alteration of their habitat in the short term. The utility of such data for prediction of the resiliency of the native soil population to long-term perturbation is frequently limited. To achieve the latter objective, soil samples should be incubated for a sufficient time period to allow the microbial community to adjust to the newly imposed conditions, particularly if the chemical or physical properties of the soil are being altered.

The limitations resulting from failure to allow community development in laboratory study are exemplified by evaluating the impact of varying soil pH beyond ranges normally detected in the soil ecosystem studied. It is frequently desirable to determine the pH range over which an enzymatically or biologically catalyzed process occurs. It could appear reasonable that the impact of variation in this parameter could readily be observed by varying the pH of one or a few representative soils. For this experiment, soil could be amended with different amounts of sulfuric acid, yielding soil samples with pH varying at set intervals over the pH range of interest. (Note that in this example, we are not considering any effect of the elevated sulfate concentration on soil biological function.) The biological activity would then be measured. The results of such an experiment are reasonably predictable. An ecological maxim is that the active microbial population in any soil is that which is best capable of coping with the prevailing soil pH. Thus, the most likely outcome of the experiment is that major changes in the soil pH will be detrimental to the soil microbial activity. The experimental data support an *a priori* conclusion that the biological community in that particular soil sample has a defined pH range in which it is capable of functioning similar to the hypothetical curve plotted in Figure 5.1. Were this be applied to our understanding of the function and resilience (sustainability), we would be lead to a conclusion that soil pH should be rigidly maintained within a rather limited range. The conclusion results more from limitations of the experimental design than actual field situations.

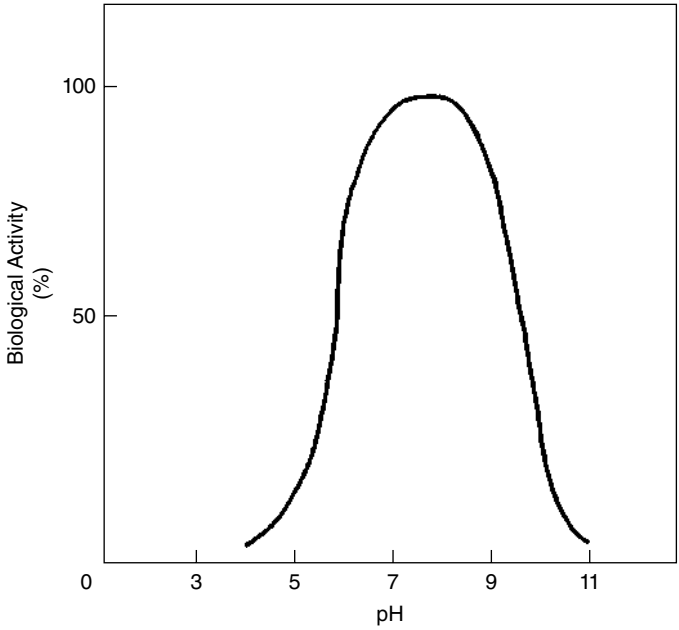

Figure 5.1 Example of variation of an hypothetical biological activity with changes in soil pH. For this representation, an optimum of approximately pH 7 with rapid declines in activity above and below this value is depicted.

In this type of experiment, the potential for development of alternative microbial communities capable of functioning at the newly imposed soil pH is overlooked. Since acidophilic or alkylinophilic microbes exist in low numbers in the original soil sample or be present in a resting state, an alternative microbial community capable of functioning under the newly established conditions can and generally does evolve. An appropriate conclusion for the experiment would be that in the short term, a significant change in soil pH would be detrimental to microbial function. An evaluation of the microbial populations developing over extended timeframes would result in a more realistic conclusion that pH does indeed result in immediate declines in activity but given time, the resilience of the existing population of microbes in the soil allows development of an alternate microbial community optimized for the newly established conditions. That is, the existence of inactive microbial species or strains capable of functioning under the newly established acidic or alkaline conditions in the original soil sample, given appropriate time for growth and induction of the requisite enzymatic activity, increases the adaptability and sustainability of soil systems.

This examination of the effects of pH on microbial populations also provides an excellent example of the complications from the interaction of soil chemical

processes on the biological response to alteration of the soil pH. Complications of data interpretation associated with adjustment of soil pH result not only from selection of the existent populations best adapted to the newly established soil pH, but also from the fact that the reaction of community to the alteration of the soil pH may be moderated due to the soil buffer capacity. Sufficient acid can be added to a soil sample to reach an acidic pH selected for a particular experimental design, but if the pH of the soil sample is measured a few hours or days after initial adjustment, it is commonly found that the pH level has returned to a value approaching the preamendment level. The impact of the added acid on the soil pH has been moderated by the soil buffer capacity (see Brady and Weil 2017) for further discussion of this process). Thus, not only is it necessary to incubate the soils for sufficient period to allow selection of acid-resistant microbial populations, but the natural tendency for the soil to return to native conditions must be considered. Changes of the soil pH during the incubation period may allow continued activity of the native population rather than development of acidophiles or more acid-tolerant organisms. Thus, to prevent misinterpretation of the laboratory studies, soil pH of the amended soils must be monitored throughout the study.

In conclusion, native soil samples may be adjusted to mimic variation in a particular soil property, but the data produced will reflect capabilities of the native soil community to cope with the change, not the capabilities of an ecologically adapted community. To achieve a measure of the latter situation, the pH-adjusted soil samples would have to be incubated for months or longer to allow the results of the selective processes to develop fully.

This consideration of microbial community reactions to changes in soil pH also provides an excellent example of the impact of the microbial functional diversity on the composition of the active portion of the microbial community. Changes in a specific soil property, such as pH or even growth of plant roots, can result in emergence of microbial species or strains better capable of functioning under the newly imposed conditions. One could envision the soil microbial populations as consisting of the fully functional individuals best adapted to the existing conditions plus those incapable of competing with their better adapted associates but fully capable of emerging as full participants in community function should the conditions change. This aspect of soil life is essential for the sustainability and resilience of soil life in its changing world. An excellent example of this sustainability/adaptability property of the system is exemplified by the response of soil microbes to short- and long-term variation in soil salinity. Stresses encountered by matric and osmotic potential fluctuations due to water evaporation necessarily induce microbial community activity and composition changes. (See Asghar et al. 2012; Chowdhury et al. 2011; Mavi et al. 2012 and Yan and Marschner 2012 for further information on soil salinity variation and microbial function.)

5.1.2.2 Evaluation of Soil Properties and Microbial Variability in Native Samples

An alternative available to overcome this potential for laboratory-generated misunderstandings of the complexity of soil community resiliency and adaptability is to study microbial population dynamics in several native soil samples that already exhibit variation of the property of concern. Microbial populations in native soils could be reasonably anticipated to have adapted to the soil site. Thus, if pH interactions with microbial activity were to be studied, it could be assumed that the microbial community existent in each soil is the one best adapted to function therein. A major caveat for such studies is the need to remember that the specific organisms active in a soil sample are those selected by the specific existing combination of soil properties, not necessarily the individual property under study. Selection of appropriate soil samples for these studies is complicated. Except under fortuitous situations, it is rare to find a series of soil sites where only one or a few soil properties vary in an otherwise constant environment. Thus, for study of native soil samples, not only is it necessary to assess the biological activity of interest, but all possible chemical and physical soil properties that could impinge upon that activity must also be quantified.

Problems in data interpretation with these field survey experiments are encountered in that statistically significant relationships may be obtained that are irrelevant. For example, the relationship may be fortuitous in that the variables being measured could have correlated with a third factor that was not considered. A potential solution to this difficulty is that once significant correlations are determined in the field, the data can be verified under more controlled conditions in the laboratory. A further means of confirmation would be to use the field-collected relationships to construct a mathematical (geostatistically derived) model of the biological activity of the soil ecosystems. Field validation of the model may provide evidence in support of the conclusion that appropriate relationships have been elucidated and quantified.

5.2 Impact of Individual Soil Properties on Microbial Activity

From a somewhat naive point of view, soil appears to present a reasonably accommodating habit for microbial growth, Mineral nutrients, organic matter (including fresh plant material), and growth factors necessary to meet microbial nutrient requirements generally exist within appropriate temperature, moisture, and pH ranges for microbial growth. Also, most soils contain microbial populations with the necessary genotypic capacity to catalyze all requisite reactions for ecosystem

development. Unfortunately for the microbe, soils also contain a variety of growth inhibitors – for instance, heavy metals, as well as organic and inorganic acids. Therefore, to fully understand biological activity in soil, the needs of the organisms must be considered in relation to the form and distribution of chemical and physical prerequisites for microbial growth.

5.2.1 Availability of Nutrients

Microbes require an energy source, electron acceptors, trace minerals, and several macronutrients plus, frequently, a variety of growth factors (i.e. vitamins and amino acids that are incorporated into cellular substituents intact) to proliferate. Although these substances are generally present in soil, the extent of microbial community development is determined by their distribution and concentrations within the microsites where the microbes reside. Therefore, the following discussion is presented with the objective of evaluating the biological requirements for growth and reproduction and the availability of these nutrients to soil communities. (Details of several microbial mechanisms for energy recovery and incorporation of micro- and macronutrients into biomass are presented in Chapter 4.)

5.2.1.1 Soil Microbial Nutrient Resources

Energy source: The primary energy supply for all living systems is solar energy. Plants and microbes (photoautotrophs) convert this energy into the chemical energy used by chemoautotrophs (heterotrophs) organisms to support cellular growth and replication. Note that due to the diversity of metabolic capacities of soil bacteria, a portion of the soil microbial community is also capable of taking advantage of the nonsolar-derived energy sources available in soil including reduced minerals (e.g. S^0, Fe^{2+}) and products of cellular respiration (e.g. NH_4, H_2). Typical energy-yielding transformations of these substances are:

$$NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$$

$$H_2 \rightarrow H_2O$$

$$Fe^{2+} \rightarrow Fe_3^{3+}$$

Although there are many opportunities for chemolithotrophic (energy recovery from oxidation of inorganic chemicals) metabolism in soil, the bulk of the soil community is driven by the oxidation of organic substances. Heterotrophic organisms primarily use photosynthetically synthesized organic carbon, most commonly entering the soil ecosystem as root exudates, dead or decaying plant biomass, or soluble organic substances leached into the soil from surface litter.

Plant biomass can also become available to soil microbes through anthropogenic intervention such as through soil amendment with manure or composts or with a variety of anthropogenically modified and industrially produced waste materials (e.g. petroleum by-products and industrial wastes). Because the energy contained in newly synthesized plant biomass is easily recovered by soil microbes, sites receiving these substances could be likened to oases within a desert of biological activity. This conclusion is based on the fact that in the absence of the reasonably constantly supplied source of carbon and energy produced by growing plants, soil microbes generally are left to metabolize the components of soil humus. This latter nutrient source is of lesser quality than the fresh, green plant biomass. Previous microbial activity has reduced the concentrations of easily metabolized organic matter. Thus, the substance remaining, the humus, is enriched in more complex organic compounds and microbial products.

5.2.1.2 Terminal Electron Acceptors

Recall that a primary chemical maxim is that all oxidative reactions must be balanced by reductive reactions (see Chapter 4). The biological reactions catalyzed within soil are not an exception to this rule. The most common electron acceptor in soil is molecular oxygen, which is reduced to water. For facultatively anaerobic organisms, oxygen and organic are terminal electron acceptors whereas the strictly anaerobic bacteria reduce organic carbon acceptors such as organic acids. Some aerobic organisms growing under anoxic conditions reduce nitrate or nitrite (denitrification) or sulfate (dissimilatory sulfate reduction) (see Chapter 4 for further details of these processes).

5.2.1.3 Macronutrient Sources

Following energy sources, the next most abundant requirement for cellular growth and proliferation in soil is a variety of macronutrients (i.e. carbon, nitrogen, phosphorus, and sulfur supplies). These substances are the major building blocks for cellular biomass. Carbon and nitrogen are generally derived from the mineralization of plant biomass, whereas phosphorus and sulfur are not only components of biomass but are also present in high concentrations in soil minerals. Thus, pathways of carbon and nitrogen mineralization are frequently interlinked whereas phosphorus and sulfur supplies and transformation in soil also generally involve both transformations of organic compounds and of soil minerals.

5.2.1.4 Determination of *In situ* Limiting Nutrients

Evaluation of nutrient limitations in soil communities is usually focused on energy or macronutrient supplies. Typically, documentation of such limitations entails amendment of soil samples with a nutrient that is suspected to control microbial metabolic activity and measurement of microbial respiration and/or

growth. If the soil amendment is utilized by the soil microbes, a variety of responses of the microbial community may be observed. An idealized depiction of the type of data that could be recovered from such studies is presented in Figure 5.2. If the microbial population was not controlled by the soil amendment or an alternative factor limited biological activity, curves of type A could be observed. For example, assume that the objective of an experiment is to determine the primary limitation to aerobic microbial respiration in a soil collected from a swamp. Observation that metabolizable fixed carbon compounds occur at extremely low levels in the soil leads to development of the hypothesis that microbial populations are carbon limited.

A typical experiment to test the hypothesis could be to amend the soil with an easily metabolized carbon source, such as glucose, glycerol, or acetate, under the conditions occurring in the field site and observe the effect of the amendment on microbial respiration or growth. When this is done under the anoxic conditions of a swampy soil, no change in microbial growth of culturable aerobic bacteria may be observed. An easy explanation is that the carbon source was not the limiting

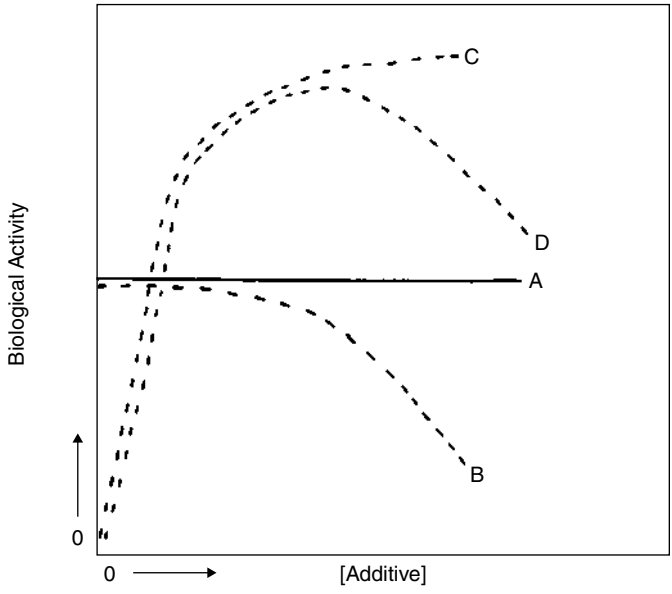

Figure 5.2 Hypothetical curves describing response of soil biological activity to amendment with proposed growth limiting nutrients. A: The added substance is not controlling cell replication. B: The additive is nonlimiting and toxic to the biological community. C: The substance was present in the soil sample in growth controlling concentrations. D: The additive is growth limiting, but it is also toxic in elevated concentrations.

nutrient controlling microbial growth. In this situation, the absence of the electron acceptor, free oxygen, likely precluded the rapid catabolism of available carbon and resultant multiplication of the aerobic organisms. Thus, prior to soil amendment, the low levels of organic substrate were sufficient to support the amount of microbial respiration occurring therein at a maximal level. Oxygenation of the system or provision of an alternative electron acceptor (e.g. nitrate) could result in development of a carbon-limited system. The probable primary delimiter of microbial activity (a terminal electron acceptor), in that case, would be relieved by addition of free oxygen or nitrate, (i.e. the available energy source could become the prime candidate or primary obstacle to maximal microbial activity).

An alternative to this neutral response of soil amendment is the situation described by Curve B in Figure 5.2. These data reflect the condition where increases in soil concentrations of a hypothetical controlling nutrient results in inhibition of overall microbial respiration; that is, the amended substance is toxic at higher concentrations. An example of this effect of soil amendment is provided by the frequently observed result of addition of a variety of aromatic compounds to a soil sample. In low concentrations (typically less than 0.1% (wt/wt)), microbial respiration may be stimulated by such compounds, such as catechol or benzoic acid, but as their concentration is increased, microbial respiration is inhibited. Thus, if maximally tolerated levels of these substances already exist in a soil site, further amendment with them inhibits cellular respiration.

More easily interpreted examples of relief of metabolic limitations are exemplified by Curves C and D. In these situations, the test compound controls microbial activity. As its concentration is increased, microbial activity or respiration increases until a plateau is reached. No further increase in activity is detected. At this point, another soil factor has become limiting to the microbial community or population studied. At maximum concentration, the amendment may have no further impact on soil activity (Curve C) or it may become inhibitory (Curve D), as was described above by Curve B. Note that with time, the microbial respiration may again increase. This change in respiration rate would result from induction of new enzyme synthesis by exiting microbes, or given sufficient time, new microbial growth. The prior leveling of the activity could be interpreted to result from the enzyme or microbial populations being the factor preventing the microbes from utilizing the more abundant nutrient and energy supply. Of course, other nutrients or soil properties (such as space for microbial growth) could also have precluded further increases in microbial respiration and/or growth.

Fluctuations in soil microbial populations following clear-cutting of a forest provide an excellent example of the impact of nutrient availability on biological activity *in situ* (e.g. Lundgren 1982). Generally, clear-cutting of the forest initially results in an increase in soil microbial biomass. This microbial response results from the decomposition of excess organic matter, both living and that previously

accumulated in soil as plant residues. These substances become available to the microbial community through destruction of the aboveground portions of the plant biomass. Roots die and carbon compounds from the debris produced by the cutting of the trees leach into the soil profile. Furthermore, machinery and logging traffic on the soil surface results in disruption of soil structure, which enhances availability of any organic matter occluded in soil aggregates.

Three microbial communities can be described for this system in transition: (i) the native forest soil community existing prior to anthropogenic intervention, (ii) an expanded community supported by the release of plant debris during logging (a disturbed soil community), and (iii) a community that develops in response to the newly established ecosystem properties (bare soil). In a nutrient-controlled situation, the disturbed soil community would exhibit maximal population density, whereas the bare soil reflects a nutrient- and energy-limited community structure reflective of the denuded, minimally productive aboveground ecosystem. With time, a new microbial community reflective of the newly established ecosystem emerges. Similar situations could be proposed to exist during the annual cycle of crop growth in a wheat field. In the spring season, the young succulent plants would be providing carbon compounds to the microbial community through root exudates. The nature and quantities of the nutrients leaking from the roots change during the growing season, resulting in alteration of the nutritional pressures imposed on the microbial community. The potential mid-season reduction of root exudates would be followed by harvesting of the crop when the plant debris and decaying roots would become the primary controller of carbon metabolism.

5.2.1.5 Carbon Limitation of Microbial Activity

Examination of the occurrence of macronutrients in a soil sample (carbon, nitrogen, sulfur, and phosphorus) and their ratios in soil microbial communities reveals that of these substances, carbon resources are generally the primary controller in soil microbial community development. Soil microbial biomass contents of soils usually correlate with soil organic carbon contents and are generally stimulated by fixed-carbon amendments (e.g. Knapp et al. 1983; Schniirer et al. 1985). The capacity for soil microbes to utilize efficiently and effectively the pools of metabolizable carbon contained in soil and to be easily stimulated by soil amendment with such materials has been adequately and frequently documented. Similarly, it is commonly noted that nitrogen is rarely limiting to soil microbes, but the active incorporation of nitrogen into soil microbial biomass may cause nitrogen to become limiting to the plants growing in the ecosystem. The low quantities of sulfur and phosphorus required by soil microbes may be supplied through mineralization of plant biomass or through solubolization of soil minerals. Again, although these macronutrients are commonly present in growth-controlling concentrations for higher plants, soil generally contains adequate sulfur and phosphorus pools for microbial development.

Growth factors: Growth factors are organic compounds required by the cell in small quantities – for example, vitamins and amino acids, purines, and pyrimidines. They are incorporated into the cell intact. It must be noted that if sufficient concentrations of these materials exist in soil, they may also be used as carbon, nitrogen, and/or energy sources. For example, a large proportion of the bacteria growing in the rhizosphere are capable of mineralizing amino acids to meet these nutrient and energy requirements.

Species requiring growth factors are more common in soil than are those capable of synthesizing them *de novo*. This implies that common sources of growth factors must be present in soil. Four reservoirs of microbial growth factors can be readily identified.

- *Other organisms growing in soil:* Each microbe in soil is capable of synthesizing a portion of the growth factors that are necessary for its biomass production (prototrophs). A microorganism may produce more than is required for internal consumption. Excess growth factors thus may leak from the cell into the suspending milieu. This cross-feeding of nutrients (utilization of growth factors leaking from a producer cell to cohabitants of the habitat) is frequently observed when viable cells are grown in petri plates containing vitamin- or amino acid-free media that support development of heterotrophic bacterial colonies. Small satellite colonies develop around the prototrophic bacterial colonies. Alternatively, supplies of the growth factor become available to auxotrophs (those requiring the growth factor) upon death and lysis of the neighboring cells.
- *Root exudates and sloughed cells:* Plants commonly leak a mixture of growth factors (including vitamins and amino acids) from their roots. Furthermore, as the root extends through soil, packets of nutrients as sloughed cells are released from the root surface and consumed by the soil microbial community.
- *Anthropogenically supplied organic substances:* Organic matter incorporated into the soil (waste disposal, sludges, composts, and accidental spills) also contains a variety of vitamins as well as macronutrients. These substances are found within the residual cells of the composted vegetative materials as well as in the associated microbial community. Lysis of the plant cells and death and lysis of the microbial cells liberates the nutrients for consumption by the soil microbes.
- *Whole plant and animal cells:* Predators and parasites receive their growth factors directly from the host cells.

The dominant organisms present in an ecosystem are usually those requiring a few specific growth factors, at most. More fastidious metabolic needs are usually associated with the microbial populations associated with living plants or animals (e.g. rumen or intestinal organisms, and root symbionts). In these latter situations, an organism capable of scavenging growth factors from its environment clearly has an advantage over one that must expend energy to synthesize the factor, but once the available supply of the vitamin or amino acid becomes limiting,

the roles are reversed. In a soil system barren of growth factor resources, prototrophs are better competitors than are auxototrophs. An exception to this generalization is associated with those microbes that are capable of forming resting structures, such as endospores. For these microbes, once the easily recoverable supply of growth factors is depleted and their competitive advantage is lost, they form a resting structure wherein they persist until growth conditions improve.

5.2.1.6 Trace Metal Supplies in Soil

A variety of metallic ions are also required for microbial growth. Examples of universally required cations are potassium, iron, and magnesium, whereas most living cells need calcium, copper, and zinc. Cobalt is likely required by most organisms for synthesis of vitamin B_{12}. Molybdenum is required for nitrogen fixation and nitrate metabolism. As would be anticipated by the predominance and variety of mineral components of soils, except for molybdenum, these metals are generally not limiting to soil microbes.

5.2.2 Soil Water

Water is an essential contributor to the growth of soil microbes. Water not only provides an essential medium for growth of microbial populations, but it is also a primary participant in a variety of cell processes. The major roles for water in soil can be summarized as follows.

- *Water molecules are reactants in essential metabolic processes:* Water is a reactant in hydrolysis reactions (for example, cleavage of cellulose into glucose and proteins into amino acids) as well as hydroxylations, such as the hydroxylation of picolinic acid and other pyridine ring-containing compounds (e.g. see Dagley and Johnson 1963; Hirschberg and Ensign 1972; Hochstein and Dalton 1967; Tate and Ensign 1974).
- *Affects gas exchange in soil:* Soil pores are filled with air and water. If all pores are water-filled, the soil is waterlogged or saturated. Since gaseous diffusion is greater in air than in water, the greater the water content of a soil, the higher the probability of its becoming oxygen limited or even anoxic.
- *Affects microbial nutrient supply:* Water is the transport medium of microbial nutrients to the cell and of waste materials away from the cell. For nonwater-soluble nutrients, water serves as the transport medium for the extracellular enzymes or solubolizing agent to reach the substrate and convert it into water-soluble products or forms. Extracellular enzymes are generally involved in the cleavage of complex polymers into water-soluble subunits, whereas surfactants increase the availability of hydrophobic materials (such as lipoidal substances) to microbial cells.
- *Affects soil temperature:* The higher the soil water content, the more resistant is that soil ecosystem to temperature fluctuations. For example, soil temperatures

rise more slowly during daily cycles in moist or water-saturated soils during the spring than occurs in the desiccated or air-dried soils of midsummer.

- *Serves as a growth medium for microbial colonies:* Microbes function in the water layer on soil particles or within soil pores. Even in a seemingly dry soil sample, a layer of water must exist surrounding microbial microcolonies and soil particles to support a viable soil community. In reality, although soil is predominantly a particulate system, microbial life forms contained therein live in an aquatic world. This water-saturated system may consist of a microfilm a few microns thick or it may extend throughout the soil micro- or macropore system. In either case, for the microorganism to function properly, it must be totally bathed in water.

5.2.2.1 Measurement of Soil Moisture

Standard techniques for quantification and expression of soil moisture levels are a necessity to the development of predictive mathematical models of the impact of soil moisture variation on soil processes and to the provision of a basis for comparison of moisture effects on biological activity in contrasting soil types. The procedure should be easily conducted and reproducible, as well as measure an aspect of soil moisture that directly affects soil microbial activity.

Traditionally, soil moisture has been reported as gravimetric water content [ω, g $H_20\,g^{-1}$ dry soil (usually dried to a constant weight, such as at 105 °C)], and volumetric water content (θ, cm^3 $H_2O\,cm^{-3}$ soil). These two means of expressing soil moisture are related to the soil bulk density (p_b, g solids cm^{-3}) by the following relationship:

$$\theta = \omega\rho_b \tag{5.1}$$

A third term encountered on a perhaps all too frequent basis is g $H_20\,g^{-1}$ wet soil. Use of the latter term is particularly distressing. It provides some means for comparison of soils data when a single soil type is considered, but no commonalty for evaluation of results between soil types exists.

Although gravimetric and volumetric water content are frequently used to measure soil moisture by soil microbiologists, their use is somewhat problematic in that neither value describes the actual availability of water to the soil microbial community. Furthermore, these values do not vary independently of soil type. Thus, comparable gravimetric or volumetric moisture values in two different soil types do not represent comparable water conditions impacting the resident microbial communities. Although a relationship between soil moisture and a specific microbial activity can be derived for a single soil type using these values, it is not possible to determine the generalized principles behind the relationship.

A partial solution to this difficulty is to use water potential as a measure of water availability in soil pores. Water potentials – measurements with a long history of use by soil scientists in general – are governed by surface tension forces and the

radius of the menisci of the water. Thus, values can be assessed by principles of capillary rise. The mathematical expression of this relationship is as follows:

$$\tau = 2\gamma / r \tag{5.2}$$

where r is the equivalent or effective capillary radius, τ is the suction necessary to absorb the water, and γ is the surface tension of water. Note that with this relationship, as the equivalent capillary radius becomes smaller, less water is present and the suction necessary to absorb the water increases. Equivalent expressions of suction are as follows:

$$1\,\text{bar} = 10^6\,\text{dyn}\,\text{cm}^2 = 100\,\text{kPa} = 1022\,\text{cm}\,\text{H}_2\text{O} = 75\,\text{cmHg} = 0.987\,\text{atm}$$

Common water potential values found in the soil microbiology literature are the moisture content of soil at −0.3 bar (field capacity). The basic advantage of this means of assessing and expressing soil water content is that it is based on the energy required to removed the water from the soil matrix. Water adjacent to soil particles requires more energy to remove it from the soil than does the water more distant from the soil particles (see Brady and Weil (2017) for a more complete description of water distribution in soil).

Another useful expression of soil moisture is water-filled pore space (Linn and Doran 1984). Soil microbes exist in a mixed soil air-water system. In reality, it is the degree of displacement of air with water within the soil pore that combines with the rate of microbial respiration to produce anoxic or anaerobic microsites within the soil profile (see Skopp et al. (1991) for a detailed study of this process). Complete displacement of air with water restricts diffusion and thereby hastens imposition of oxygen-limited conditions. When moisture approaches or exceeds field capacity, the percent soil pore space filled with water or air becomes a better indicator of aerobic vs anaerobic microbial activity than does water content or water potential. Use of percent water-filled pore space to express soil moisture levels is practical since it only requires knowledge of the soil bulk density and the gravimetric soil water content. (The particle density of mineral soils is assumed to be $2.65\,\text{Mg}\,\text{m}^{-3}$.) The percent water-filled porosity is defined by the following relationship:

$$\%\text{WFP} = \left(\theta_v / \text{TP}\right)100 \tag{5.3}$$

where θ_v is equivalent to the product of the gravimetric water content *(ω)* and the soil bulk density (ρ_b) and TP is the percent total porosity ($1 - \rho_b/\rho_p\,p_p$) (100), where ρ_b is the soil bulk density and ρ_p is the soil particle density. For aerobic processes, maximal activity is detected at a percent water-filled porosity of about 60%. Below 60%, water limits activity (insufficient moisture exists to provide the contiguous surface film required by the soil microbes), whereas above 60% aerobic microbial

activity decreases (apparently due to reduced oxygen availability). Caution is necessary in use of these generalized figures. Neilson and Pepper (1990) found discrepancies in use of percent saturation or water-filled pore space to characterize soil aeration, especially when soils of differing bulk densities or those receiving particulate organic matter amendments are compared.

The effect of water in a heterogeneous system can also be expressed as water activity. This measurement of moisture is most commonly used in food storage to describe water availability. Water activity is related to the equilibrium relative humidity (ERH) as follows:

$$A_w = \mathrm{ERH}/100 \tag{5.4}$$

The difficulties with using water activity in soil microbiological research are the complications associated with its measurement. Long incubation times in a chamber where the moisture level of the test sample is allowed to equilibrate with the chamber atmosphere are usually required. Data can be extremely variable. Water availability affects species diversity, survival, movement, and activity of microbes. Of all of the expressions that may be used to define the moisture conditions under which the soil microbe is abiding, water activity is probably the most useful. Unfortunately, it is also the most difficult and time-consuming (i.e. impractical) measure of soil moisture.

Impact of moisture variation on soil microbial activity: Total microbial activity varies from nearly nonexistent levels at low water availability to a maximal level under optimal soil air/moisture mixtures (Figure 5.3). Typically, soil bacteria are capable of functioning under seemingly severe soil moisture tensions. For example, Wilson and Griffin (1975) found that respiration of a mixed bacterial population in soil was maintained at a reasonably high level between −8 and −30 bar, but was negligible at −50 bar. Similar results have been reported by Wildung et al. (1975) and Knight and Skujins (1981). As soil moisture levels are saturated and free oxygen supplies are depleted, overall activity again declines. A generalized curve (Figure 5.3) is applicable to the summation of microbial respiratory activities and to a certain degree individual processes. In the latter situation, the points of minimal or optimal activity are characteristics of the microbial process being studied. Obligatorily anaerobic processes clearly are optimized under saturation conditions.

Tolerance to restrictive soil moisture levels varies with major microbial groups as well as with individual microbial species. Generally, bacteria are more exacting than are actinomycetes and fungi. Thus, the latter organisms tend to predominate in drier soils. This observation results at least in part from the capacity of the latter groups of organisms to form resting structures. Robertson and Firestone (1992) postulated that bacteria may use extracellular polysaccharide to enhance survival under

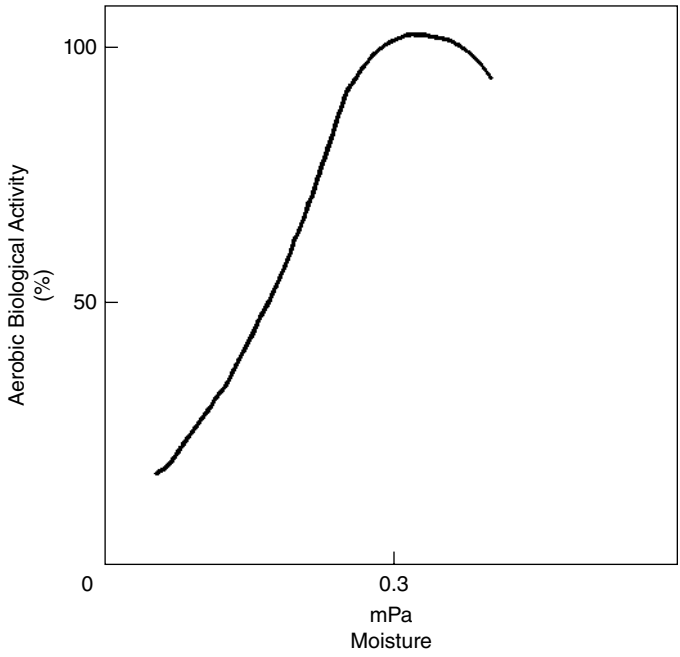

Figure 5.3 Variation in aerobic biological activity in soil. In this hypothetical example, optimal moisture is approximately 0.3 mPa.

moisture-limiting conditions. A purified extracellular polysaccharide from a *Pseudomonas* species was shown to contain severalfold its weight in water at low moisture tension. Within the realm of the bacteria, a gradient of tolerance is noted to occur. For example, ammonium tends to accumulate in dry soils due to the ammonifying microorganisms being less sensitive to the low water activities than are nitrifiers.

Selection of microbial populations with increased resistance to moisture limitations in chronically arid soils was demonstrated by Knight and Skujins (1981). These workers used adenosine triphosphate and soil respiration measurements as indicators of microbial biomass and activity. The impact of variation of soil moisture between −2 and −100 bar tension on microbial activity in two arid soils and a subalpine forest soils was evaluated. The microbial activity increased in arid soils between −2 and −20 bar soil moisture, whereas the optimal moisture in the chronically wetter forest soils was −2 bar and activity continually decreased below that level. Thus, it was concluded that a characteristic microbial populations capable of managing the prevailing environmental conditions had developed in each of the extreme environmentally different soils studied.

It is important for the student of microbiology to understand clearly what these observations reveal about the function of the soil microbial community and at least as importantly, what is not stated. The positive side of this observation is that it follows that as long as the soils are not so dry as to preclude biological function, an appropriate microbial population does develop. The negative aspect is that it is not implied that this microbial population selected for growth under restricted soil moisture is as active as a comparable population growing under less stringent conditions. That is, were microbial respiration to be measured under limited soil moisture and at optimal soil moisture, even if the organisms in each situation were absolutely optimized to their environment, less respiration would be found in the low-moisture soil. Practically, this means that one should not anticipate as much organic matter to be decomposed in desert soils without irrigation as could occur in an irrigated field of the same soil.

The impact of excessive soil moisture (flooded or chronically waterlogged conditions) on soil microbial activities is more predictable than situations observed in habitats experiencing a shortage of free water. Interactions of soil moisture levels with availability of molecular oxygen become evident in the flooded or chronically waterlogged soil sites. A traditional division of the microbial realm has been separation by oxygen tolerance (anaerobes, facultative anaerobes, and aerobes). Flooded soils are not necessarily oxygen free, but should available free oxygen supplies be depleted, anaerobic and facultative microbial populations are stimulated while obligate aerobes die or enter resting stages. The most dramatic effect of institution of anaerobic conditions in soil is the induction of facultative (e.g. fermentations), anoxic aerobic metabolism (e.g. denitrification), or strictly anaerobic processes (e.g. methanogenesis). Organic acids may accumulate, nitrogen oxides could be evolved, and ultimately, methane generation or sulfate reduction may occur. Practically, anoxic soils may be recognized by precipitation of metal sulfides, or evolution of an amine or sulfide odor. Characteristics of the microbial community are dependent upon the duration of the flood period and the nature of electron acceptors present. Methanogenic bacteria require development of highly reducing conditions, whereas fermentative organisms are less stringent (see Chapter 4).

As stated above, a flooded soil is not necessarily either anoxic or highly reducing. Water movement through the environment as well as diffusion of atmospheric gases into the system may prevent exhaustion of free oxygen. This phenomenon was shown in a study of the muck soils of south Florida (Tate 1979). With flooding of the soils, an increase in aerobic bacterial populations was initially observed (Figure 5.4). The soils were flooded to maximum depths of approximately 31 cm. As the free oxygen and easily metabolized carbon source levels declined, a parallel reduction of aerobic bacterial populations occurred. Thus, were the experimenter assuming that flooded and anoxic conditions were equivalent, during the initial

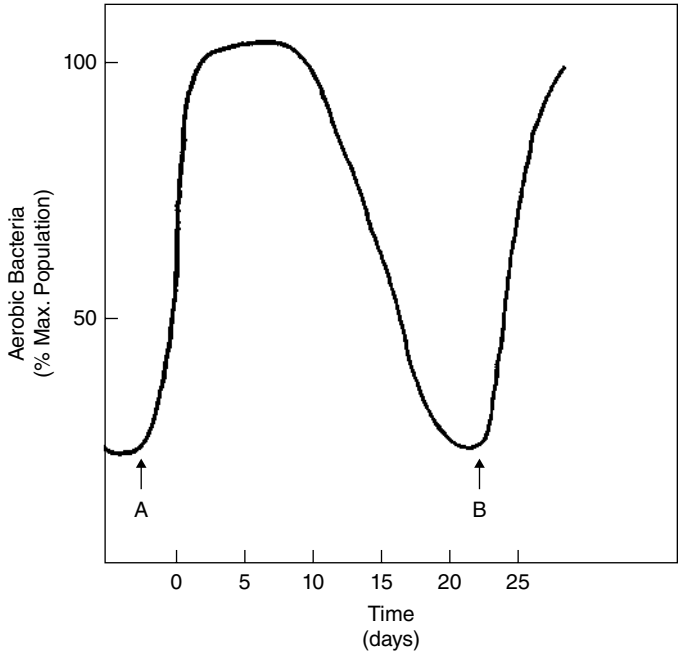

Figure 5.4 Effect of flooding a dry histosol on aerobic bacterial populations. Soils were flooded to a maximum depth of approximately 31 cm commencing on day 0 (arrow A). Arrow B indicates the time of drainage of the flood waters. *Source:* Adapted from Tate (1979).

portion of the study, a behavior of the soil microbes contrary to what occurred *in situ* would have been expected.

5.2.2.2 Soil Systems with Fluctuating Soil Moisture

Further underlying assumptions or perhaps attitudes that must be developed in relationship to understanding the effect of soil moisture on the microbial community are (i) the moisture level of a particular site tends to be continually changing and (ii) moisture effects on biological activity are interactive with other system properties.

Moisture fluctuation may be on a short-term, localized basis as would be found (i) with daily impact of temperature variation and water evaporation, (ii) following a heavy rainfall, or (iii) due to seasonal flooding, as occurs in a wetland rice soil. For example, microbial biomass fluctuations can reveal the short-term effect of soil moisture levels induced by precipitation (Clarholm and Rosswall 1980). Precipitation caused increases in microbial biomass lasting one to two days, even in nonmoisture-limited soil. This observation suggests that although data descriptive of macrosite soil moisture tensions support a conclusion that the microbial populations are not

limited by this soil property, it is microsite discontinuity in moisture films covering soil particles that determines the rate of change in and ultimate level of microbial population densities. In flooded rice field soils, anoxic conditions induce anaerobic microbial populations during the somewhat long term of flooding. Draining of the site following harvest or for establishment of seedlings induces aerobic and facultative microbial populations. Strict anaerobes may be further limited by the flow of oxygenated water through the system. In that situation, a mosaic develops in soil, with pockets of anoxic soil interspersed with oxygenated soil.

Note that soil systems are described as containing a mixture of aerobic and anaerobic micro- or macrosites, *not* "a partially aerobic system." By definition, each individual soil microsite is either anaerobic (anoxic) or aerobic. "Partially aerobic" is an inappropriate description in that "aerobic" indicates the presence of oxygen in the system at the location of the soil microbe, which is an all-or-none state. Even if only a few molecules of oxygen are present per cubic centimeter of soil, the site is still described as being aerobic.

The interactive nature of soil moisture with other soil properties is documented by examining wet/dry cycling on soil organic matter availability as well as salinity impacts on microbial activity (see Rousk et al. 2011; Setia et al. 2011). Variation of soil moisture with time not only affects the nature of the microbial population present in the soil, but it may also alter the quantity of nutrients or the physical structure of the microbes' environment. Cycling of soil moisture liberates occluded organic matter through such processes as disruption of soil aggregate structure (for example, see Asghar et al. 2012) or by death of the more stringent microbial populations. Therefore, microbial oxidation of soil organic matter is greater in soils with fluctuating moisture than in constantly wet or dry conditions (e.g. see Lund and Goksoyr 1980; Orchard and Cook 1983; Reddy and Patrick 1975; Sorensen 1974; Thiemann and Billings 2012). Jager and Bruins (1975) noted that the loss of carbon due to microbial respiration in a soil experiencing approximately 60 wet/dry cycles was also proportional to drying temperature, with greatest loss (31.2%) in soil dried at 85 °C and least (18%) in soil dried at 35 °C. Drying resulted in an ultimate reduction in microbial population densities, but the highest population densities were found in soil dried at 85 °C, suggesting that more organic matter was made available to support microbial growth by the more drastic treatment. A practical implication of this observation relates to maintenance of soil organic matter levels in mineral soils and the minimization of subsidence of histosols (see Tate 1980). In both situations, maintenance of the native organic matter levels is desirable for preservation of the ecosystem and/or encouragement of optimal soil structure (see Tate 1987).

An indirect effect of declining soil moisture is the impact of osmotic pressure on biological activity. Generally, the osmotic potential of water is less important than water availability, but in some localized or transient situations, variations in osmotic

potential emerge as significant controllers of microbial growth. Potentially lethal or inhibitory salt concentrations may be the product of microbial metabolism (e.g. mineral dissolution through acidification of microsites) and chemical equilibria (e.g. ionization of water-soluble salts), as well as physical phenomena (evaporation). Localized problems are associated with areas of salt encrustation due to water evaporation; saline waters such as are found in some irrigation systems, around saltwater bodies; or in association with saline soils. Transient difficulties may be associated with severe desiccation of soil.

Major impacts of osmotic pressure are likely averted in soil because of the fact that reduction of soil moisture results in induction of cellular survival adaptations (for example, sporulation). Also, in regions with chronically saline soils, selection of organisms with increased resistance to pressure changes may occur. For a discussion of microbial adaptations to osmotic changes, see Csonka and Hanson (1991).

5.2.3 Aeration

A major role of molecular oxygen in soil is as a terminal electron acceptor. As was indicated in Chapter 4, energy is produced during electron transport to oxygen. In fact, oxygen is the highest energy-producing electron acceptor. Thus, the greatest cell yields are produced by the total oxidation of an organic carbon-containing substance to carbon dioxide and water. This important function of molecular oxygen in aerobic metabolism is augmented by its direct incorporation into molecular structures of such compounds as aromatic ring-containing substances and alkanes by oxygenases. The significance of aerobic metabolism to the soil microbial community is underscored by the observation that most microbes in surface soils are aerobes. Exceptions are soils that are consistently flooded, such as bog soils.

Soil oxygen tension is controlled by gaseous diffusion rates as well as by the respiratory activity of the soil biota. Since diffusion is a relatively slow process, it is not unusual for the rate of consumption of this essential gas to be greater than its rate of replacement. Thus, free oxygen tensions are rarely too high for soil aerobes, but clearly soil oxygen levels may be too low for obligate aerobes.

Indirect limitors of soil oxygen are soil moisture and the concentrations of metabolizable organic matter. As was indicated above, moisture affects soil oxygenation in that the greater the soil moisture, the fewer air-filled pores exist, and the slower the diffusion rate. Decomposable organic carbon availability controls the rate of oxygen consumption indirectly in that as this fixed carbon is oxidized, free oxygen may be depleted. With an influx of easily metabolized organic matter, it is not unusual for anoxic conditions to develop. Examples of practical consequences of oxygen depletion due to soil microbial respiration are as follows.

- Oxygen depletion in cropped soils after a heavy rainfall event can result in crop damage.
- After a heavy rainfall, with the presence of nitrate and suitable carbon sources, significant losses of fixed nitrogen from soil can result from induction of denitrifiers.
- Spills of easily decomposable organic matter in soil or incorporation of readily metabolizable organic matter can result in depletion of free oxygen supplies thereby resulting in ecosystem disruption through oxygen starvation of plant roots, death of small animals, earthworms, nematodes, mites, and so forth.

As the free oxygen in soil is depleted, a number of predictable changes in the microbial activity occur. When the soil oxygen tension has been reduced to less than 1% (v/v), the microbial population appears to shift from being predominantly aerobic to anaerobic. With the development of a reducing atmosphere, growth yields decline since the energy yielded per mole of fixed carbon oxidized anaerobically is far less than that produced from aerobic respiration (see Chapter 4). (Note that dissimilatory respiration of aerobic bacteria [denitrification and sulfate reduction] cell yields approximate those with oxygen as the terminal electron acceptor since a cytochrome based electron transport processes occurs and the carbon substrates are oxidized completely to carbon dioxide, water, and, ammonia.) Fermentation results in accumulation of incompletely oxidized carbonaceous substrates (organic acids, amines, etc.). When highly reducing conditions develop so that methanogenesis and sulfate reduction occur, complete mineralization of fixed carbon substrates again occurs.

5.2.4 Redox Potential

It is difficult to separate the impact of redox potential from oxygen tension effects experimentally. To artificially raise the redox potential in the absence of free oxygen, it is necessary to add oxidizing agents, which are generally toxic to microorganisms. Thus, it must be noted that changes in soil redox potential are related to changes in oxygen levels. If organic matter is added to soil, oxygen is depleted and the potential drops – at times quite precipitously. This is a microbial reaction since inhibitors of microbial activity prevent both oxygen depletion and development of reducing conditions – which is somewhat logical since in an actively respiring community, oxygen is used as an electron acceptor.

As has been alluded to above, the occurrence of a variety of microbial processes is related to specific redox potentials. Some of these are as follows.

- Aerobic carbon oxidation >0.2 V
- Denitrification 0.15 to 0.2 V
- Methanogenesis −0.2 to −0.1 V
- Sulfur reduction −0.2 to −0.1 V

Although these data suggest a somewhat mutually exclusive nature of microbial activity in soil, it is not unusual to observe these processes occurring concurrently across a landscape of limited size. Again, this results from the heterogeneity of the soil system. Two examples of this situation (variation of redox potential in a flooded soil and within the profile of a drained histosol) are provided in Figure 5.5. In both situations, an oxygen-rich, high redox potential region overlays an anaerobic portion of the soil profile. A region of variable conditions generally occurs between these two extreme environments. Similarly, a mosaic of activities can occur in a well-aggregated soil where aerobic processes predominate on the surfaces of the aggregates and anoxic processes occur in the interior.

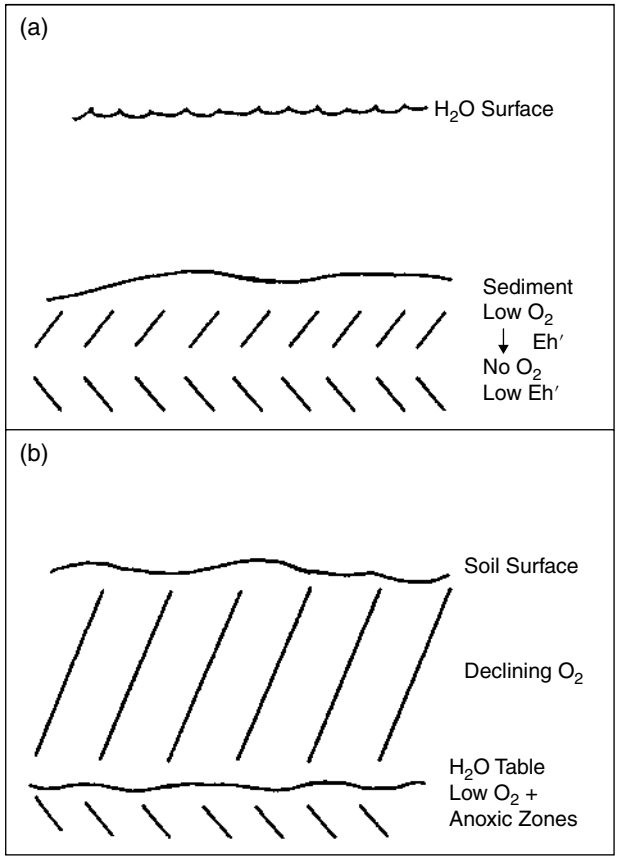

Figure 5.5 Redox potential and oxygen tension patterns in an hypothetical flooded soil (a) and a soil with an elevated water table (b).

5.2.5 pH

The microbiologist's bias tends to favor conducting experiments with soils with approximately neutral pH and the study of the microbes capable of functioning therein. Our laboratory media developed for the culture of microorganisms are so constituted, and the majority of the soil systems studied tend to fall in a pH range of approximately 5.5–7.5. Yet acidic soils constitute a major portion of the world's soil resources, especially as exemplified by soils of tropical regions and temperate forests, and acidification is a major reclamation problem in the normally near-neutral soils of the temperate region. For example, with many mining processes, metal-sulfide-bearing slags are produced. Biological activity in these waste materials results in oxidation of the sulfide to sulfate. Subsequent drainage of these acid-bearing waters presents major problems for downstream soils and receiving waters. Thus, it is imperative to understand the effect of soil pH on soil microbial community development and function.

Acidic conditions present a particularly stressful situation to the microbial cell. Organisms existing in acidic habitats could be described as being in a somewhat schizophrenic state. The internal cell pH must be highly buffered at or near neutrality, the pH optimum for internal enzymes. Thus, the effect of variation of the external pH necessitates development of cell surface and membrane-associated mechanisms to control the internal pH. Furthermore, metabolic processes occurring at the cell surface must be adapted to cope with the acidic conditions.

A variety of pH-associated metabolic problems must be surmounted by the cell. For example, cellular surface charge varies with environmental pH. This controls the interaction with soil particles as well as with charged nutrients. Furthermore, surface-bound enzymes as well as extracellular enzymes (see Chapter 6) must also be capable of functioning at the elevated hydrogen ion concentration. Maximal activity must either be expressed at the predominant pH, or enzyme synthesis rate must be modified to compensate for the reduced enzymatic activity occurring under the acidic conditions. Also, adjustment of enzyme synthesis rates may be necessary to compensate for loss of those enzymes that are denatured under acidic conditions. If the enzyme product is essential for cell function, then the organism must adjust the enzyme synthesis rate so that it is always present at effective levels.

5.2.5.1 Indirect Mechanisms for pH Limitation of Biological Activity in Soil

Nutrient concentrations and toxicity of environmental substituents are both affected indirectly by soil acidity. Trace mineral availability provides an excellent example of pH interactions with nutrient availability. Both iron and manganese are more water soluble at low pH, whereas molybdenum is precipitated at low pH.

Similarly, capacity to transport charged carbonaceous substances such as fatty acids and amino acids into the cell is dependent upon the charge of the molecules. Reduction of the soil pH below the pKa of acids results in conversion of the negatively charged entity to a neutral compound. Zwitter ions may have an overall negative charge or positive charge, or be uncharged depending upon the pH of the suspending medium. For example, glycine ($^{+}H_3NCH_2COO^{-}$) has both positive and negative charges at neutral pH. As the pH is varied, the positive and negative charges are neutralized independently. Each of these states would result in alteration of the capacity of the cell or its catabolic enzymes to catalyze transformation of these materials.

Under acidic conditions, organic acids can be toxic to microbial growth. This is not a pH effect, but is an impact of the presence of the organic acid itself in an uncharged or neutral state. Thus, accumulation of organic acids under anoxic conditions in acidic environments could be a mechanism for limiting microbial activity. This interaction of pH and anaerobic conditions may contribute to organic matter accumulation in acid swamps.

A final indirect effect of acidic soil pH conditions on biological activity relates to the potential for solubolization of toxic compounds or elements. Aluminum is more available under acidic conditions. It is not uncommon for toxic levels of this cation to be detected in acidic tropical soils.

Direct modifications of biological activity by pH variation: The direct effect of pH relates to the fact that each microbial strain has an intrinsic range of pH within which it is capable of functioning. The diversity of soil life is exemplified by the observation that microorganisms grow from about pH 1–11. Similarly, since it is to a large degree the property of an organism's enzymes that determine the diversity of processes catalyzed, a comparable reaction to environmental pH is found for these cell components. Although microbial life occurs at essentially all naturally occurring soil pH levels, each individual species (and to some extent, strain) has a characteristic pH range and optimum pH for growth. For example, *Thiobacillus thiooxidans*, a key organism in oxidation of reduced sulfur-containing compounds, grows only at extremes of acidity (pH 2–4), whereas *Nitrosomonas* sp., a key player in biological nitrification, has an optimum pH in the alkaline range and is not active below about pH 5.5. Similarly, another sulfur-oxidizing bacterium, *Thiobacillus thioparus*, grows only at near-neutral pH.

Some selection of overall contributors to biological activity results from variation of soil pH. In acid soils, the dominant activity is generally the result of the fungal community. This results from the poor growth of most heterotrophic bacteria at acid pH. An exception is with waterlogged acid soils. Here, the aerobic fungi are inhibited by oxygen limitations, so the acid-tolerant bacteria are better able to compete.

Some microbially catalyzed processes occur in soils with a specific pH range. For example, bacterial catalyzed nitrification (oxidation of ammonium through

nitrite to nitrate) is suggested to occur primarily in soils with a pH greater than 5.5. This observation results from the fact that the bacteria strains involved in nitrification belong to a small group of autotrophic bacterial species that are pH sensitive (Dommergues et al. 1978).

It can be generalized that if a process occurs over the entire pH range conducive to development of living cells, the transformations result from the activity of a variety of microbial species or strains with varying pH optima. For example, carbon and nitrogen mineralization occur in essentially any soil ecosystem where life is possible. Thus, a measure of the diversity of the microbial community is revealed by the breadth of conditions under which the organisms are able to function. This maxim relates not only to soil pH but also to other properties, such as moisture and aeration.

5.2.5.2 Conflicts Between Laboratory Observations and Actual Field Activities

Some anomalous situations have been encountered. These are associated with conditions where laboratory experiments with axenic microbial cultures yield data suggesting that the only known microbes capable of catalyzing a particular process cannot function at the prevailing soil pH of the site under study, yet the products of the reaction are detected. For example, as indicated above, acidophilic autotrophic nitrifiers have not been detected in acidic forest soils, yet nitrate is commonly found in these soils. Several explanations have been proposed to explain this seemingly anomalous observation (for example, see Dommergues et al. 1978). Alternative explanations include the following: the microbes may be growing in soil microsites with a more favorable pH. That is, the acid-limited microbes may be constrained to islands of more moderate conditions contained within a sea of restrictive soil. This situation would be more likely to occur in soils where the overall pH is near the extremity of function of the microorganism. That is, an overall soil pH of approximately 5.0 could be measured, yet microsites 0.5 pH units above or below that value may exist.

An alternative explanation to the anomalous occurrence of a pH-limited process may involve our capability of culturing the responsible microbes or even discovering the existence of microbes with appropriate capabilities. This limitation is exemplified by the increasing number of anaerobic ammonium-oxidizing (anammox) bacterial strains being isolated that are capable of catalyzing processes previously considered to be pH and oxygen limited. (See the following publications for further discussion of this topic: Gijs Kuenen 2008; Kartal et al. 2008; Schmidt et al. 2002, 2005.) Only a small portion of the microbial community in any soil can be cultured and studied in the laboratory. Techniques for the study of the vast majority of this diverse community remain to be developed. Thus, occurrence of processes in the field may be a better indicator of microbial capabilities than is the library of information collected from axenic cultures.

Lastly, a spontaneous chemical conversion of the reactants may occur outside the pH range optimal for biological activity. This is exemplified by denitrification under acidic conditions. In this situation, bacteria can be cultured that catalyze reduction of nitrate to nitrous oxide and dinitrogen under acidic conditions, but their function in the native acidic soil is questioned. The kinetics of the spontaneous chemical reaction favor that process over the biologically catalyzed nitrogen oxide reduction.

In conclusion, a variety of interactions of soil hydrogen ion concentration and soil biological process have been elucidated. Life does occur in extremely acidic or alkaline soils. The participants may vary from those under less stringent conditions, and the range of processes occurring therein may be somewhat truncated. Stewardship of acid soils and reclamation of acid-affected soils requires consideration of the reactions occurring therein and the limitations of the system. Due to the intrinsic buffer capacity of soil, management of an acidophilic community may be necessitated by the inability to adjust the pH of many soils to a more neutral status (e.g. Magdoff and Bartlett 1985).

5.2.6 Temperature

Greatest resistance to temperature extremes among living entities is found in the microbial community. The typical growth range for soil inhabitants is more restrictive than the extremes associated with other ecosystems. The typical breadth of temperatures supportive of microbial growth in soil is from approximately 0 °C to 70 °C. This contrasts on the upper side of the range to growth of bacteria in hot springs, which are capable of growth at 100 °C (Heinen and Lauwers 1981). Minimal growth temperatures have been shown to be slightly below 0 °C in Antarctic soils. With either maximum or minimal growth temperatures, the limiting factor appears to be free water. As long as the conditions allow existence of free water (either through boiling temperature elevation by augmented pressure or freezing point reduction by salt concentration), microorganisms appear to be capable of growth, albeit slow growth at the reduced temperatures.

Reduction of soil temperatures below freezing results in significant declines in microbial populations (Biederbeck and Campbell 1971). The cause of cell death is not the freezing of the cell, in that freezing itself causes only a small reduction in microbial numbers. The lethal factor appears to be associated with slow thawing of the soil. As soils thaw, ice crystals form in the microbial cells and disrupt cellular integrity. Sensitivity also relates to the growth status of the microbial cells in that recently cropped soils exhibited greater population declines due to freezing than was detected in inactive soils (Biederbeck and Campbell 1971). Furthermore a differential sensitivity of the microbial populations was noted in that bacterial population reduction was greater (92%) than that of fungi (55%), which was greater than that of actinomycetes (33%). Similar differential sensitivity of soil

bacteria to freezing and slow thawing was noted by Nelson and Parkinson (1978) in a study of a *Pseudomonas* sp., *Bacillus* sp., and *Arthrobacter* sp. Survival was dependent on soil moisture, storage time, and the thaw rate.

More typically for soil microbes, growth can be depicted as an activity range (Figure 5.6). The growth rate increases until a maximal temperature, characteristic of the organism, is reached. The maximum temperature that supports growth is determined by the sensitivity of the most fastidious enzyme essential for cell replication. This temperature sensitivity curve is a good example of the *law of tolerance* which states that a microorganism will have an ecological minimum and maximum with a range in between that represents the limits of tolerance.

Tolerance to temperature is commonly used to divide microorganisms into three classes. Psychrophiles are defined to grow optimally at temperatures less than 20 °C. Mesophiles grow best between 15 and 45 °C, whereas thermophiles are active at temperatures greater than 45 °C. Few if any psychrophiles are found in most soils. Exceptions are associated with Arctic and Antarctic sites. Mesophiles constitute the bulk of the soil microbial community, whereas most soils contain some thermophiles, even if the soils never achieve the high temperatures.

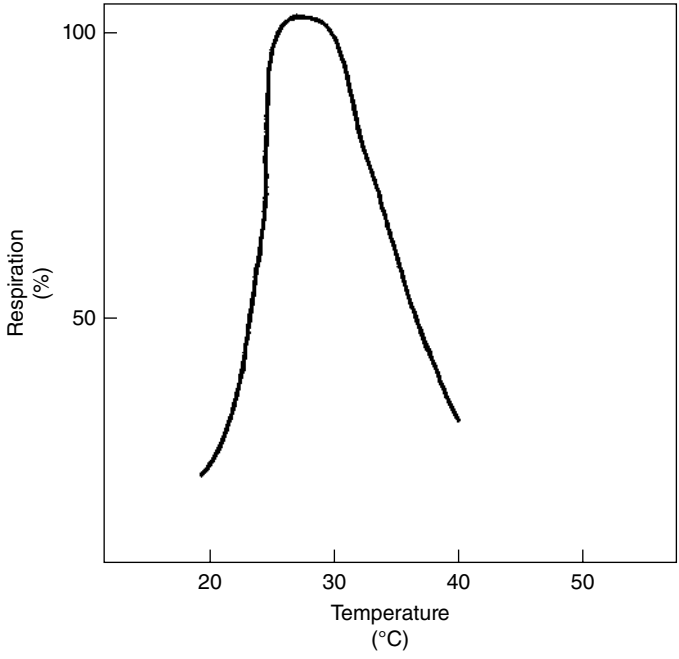

Figure 5.6 Typical response of a mesophilic bacterial population to temperature variation. An optimum temperature of 25–30 °C is depicted.

The presence of these classes of microbes assures occurrence of metabolic activity over a wide temperature range. Typically, soil biological activity increases from a minimum at or near 0 °C to a maximum and terminus point near 70 °C. The rate increase can be represented by several mathematical relationships, among which are the Arhenius and Q_{10} relationships. Historically, the latter is likely the most commonly used parameter for soil microbiological studies. The Q_{10} is equal to the reaction rate at temperature one divided by the rate at temperature one minus 10 °C, that is:

$$Q_{10} = {}^{K}{}_{T} / {}^{K}{}_{T-10} \tag{5.5}$$

Q_{10} values for biological processes generally range from 1.5 to 3.0, with a mode of approximately 2.0. Generally, if the Q_{10} is much greater than 3.0, an environmental parameter besides temperature is controlling the reaction rate. For example, Q_{10} values for petroleum decomposition at low temperatures (10–20 °C) are commonly greater than 5. At the low temperatures, catabolism of the oil is controlled by its water solubility and viscosity. These parameters dramatically change as the temperature is raised, so a disproportional impact of temperature elevation of the microbial oxidation is observed.

5.3 Microbial Adaptation to Abiotic Stress

From an analysis of the diversity of soil properties affecting microbial activity, it almost appears that life in soil would be limited at best. Yet, nearly every site on Earth supports active microbial populations. Even in situations where *a priori* considerations would predict that two factors, such as moisture and temperature, exist at limits that preclude microbial growth, microorganisms have been shown to occur in extremely specialized niches. For example, in cold dry deserts of Antarctica, cyanobacteria-dominated communities have adapted to growth between the crystals of porous rocks (cryptoendolithic) where some modulation of the extreme environmental stress occurs (Friedmann 1982; Johnston and Vestal 1991). This portends a variety of mechanisms of community adaptation to these stresses. Three reactions to stressful conditions should be highlighted.

First, if the stress is extreme, there is no adaptation. This is exemplified by soils with temperatures greater than 70 °C around hot springs or adjacent to nuclear reactor water outflows, some extremely acidic mineland reclamation sites, and soils containing excessively high metal concentrations. The existence of such ecosystems underscores the fragile nature of the biotic system and highlights the fact that at least some growth is necessary for implementation of the two following survival mechanisms.

Second, if growth is possible, microbial strains resistant or at least tolerant to the stress factor are selected. This has been observed with osmotic pressure, temperature, oxygen tension, and moisture stress. This adaptation mechanism may result in reduction of the species diversity, especially if the stress is severe, but a functional community develops. Reduction of the microbial diversity, and therefore the genetic diversity, of the community could portend enhanced vulnerability to ecological insult. Soil presents a somewhat unique exception to this principle in that the potential always exists for invasion by propagules from adjacent soils. Thus, the genetic potential required to overcome chemical or physical stress in a particular site could exist in less stressed neighboring soils. In this case, passive or active transfer of propagules from the neighboring soils may result in establishment of a new community in a site that previously lacked the resources necessary for survival.

Lastly, in some situations, the microbial community may possess the capacity to alter the stress factor, thereby relieving the impediment to growth or survival. For example, microorganisms may modulate inhibitory acidic or alkaline conditions or decompose toxic substances. Organic acids or even antibiotics may be decomposed to carbon dioxide by microbial strains resistant to their toxicity. Even nonbiodecomposible toxins such as heavy metals may be transformed to nontoxic states. They may be complexed, precipitated, oxidized or reduced, or even chelated. The products are rendered nonwater soluble, converted to water-soluble but nontoxic forms, or incorporated into a chelate complex that can be leached from the microbial habitat. Inhibitory inorganic acids may also be rendered ineffective. For example, sulfate is reduced to hydrogen sulfide, which is precipitated by metal ions, such as iron, which are common in soil. In each of these examples, homeostasis is reached by activity of resistant populations conferring benefits to the survivors of the susceptible organisms.

5.4 Concluding Comments

The nature, activity, and future of any soil microbial community are determined by the capacity of its individual members to adapt to or modify negative soil properties. Those members of the community best equipped to cope with the combination of ecological stresses are active, while more stringent organisms succumb or enter resting stages. Modification of the physical, biological, or chemical properties of the site results in induction of microbial processes to restore the homeostasis of the community. This adaptation may result in reemergence of an ecosystem resembling that existing prior to perturbation, development of a new community better suited to the newly developed conditions, or total destruction of the biological activity. The ultimate fate depends upon the genetic diversity of the populations

and the severity of the stress imposed, as well as the time allowed for recovery. Some situations may allow recovery in hours to days, whereas in others, full recovery may not be evident for decades or centuries.

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6

Soil Enzymes

Basic Principles and Their Applications

Based on the primary principles underlying microbial physiology, it seems to be logical that soil enzymatic activity would be a good measure of soil biological activity and metabolic potential. Enzymes are the primary catalysts of biological transformations. Indeed, a variety of enzyme activities have been shown to directly relate to soil biological potential (e.g. Dick 1984; Nannipieri et al. 1979; Tate et al. 1991) but frequently, little or no correlation between the levels of essential enzymatic activities and microbial processes, such as soil respiration, is observed, suggesting that at least in some soil systems, synthesis of new enzyme molecules, total enzyme present, and substrate levels for specific enzyme activities are uncoupled. Clearly this is a seemingly contradictory situation involving basic principles of biochemical processes and the importance of a variety of enzymes as controllers of microbial growth and sustenance. Thus an initial consideration for understanding enzyme activities occurring in soil and the properties of the soil controlling these essential catalysts must involve elucidation of properties of the soil system that enable this apparent incongruity to occur.

Examples of the impact of soil physical and chemical properties of soil on biochemical processes catalyzed by extracellular enzymes (enzymes released from the cell producing it in order to catalyze a reaction providing a benefit for the cell) yield insights into the complexity of the forces limiting as well as supporting biological processes in soil systems. Barriers to benefit recovery by the cell include the probabilities of (i) the enzyme encountering its substrate in the soil matrix and (ii) the product of the reaction reaching the enzyme-producing microbial cell. The first hurdle controlling this process results from fact that the microbes and the extracellular enzymes exist in a three-dimensional environment. Once outside the cell, there is no assurance that the enzyme will move in the direction toward or away from its substrate. Similarly the driving forces controlling return of the product of the enzymatic reaction to the cell are also independent of control by the microbe. Thus, the probability is large that

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the enzyme may not encounter its substrate or that the product of its reaction may not return to the enzyme-synthesizing cells.

For example, consider that a microbe is producing cellulase, the enzyme that converts cellulose to simple sugars. At first glance, considering that the cellulose may not be evenly distributed around the microbe, the enzyme molecule may not encounter the pool of cellulose existing in the vicinity of the microbial cell. Additionally, if the enzyme and its substrate interact, the glucose produced may diffuse or be transported away from rather than toward the microbe. The amount of cellulose in the soil, the total potential enzyme activity and any change in microbial population could be measured. If all or a meaningful portion of the glucose fails to reach the microbes synthesizing the cellulase, the logical, but wrong, conclusion that the cellulose is not supporting microbial growth could be reached. Everything required for the process to occur as understood from study of laboratory cultures occurred, except the spatial heterogeneity precluded return of the product to the microbes expending the energy to support the process. Were the process considered to involve retrieval of a required growth factor or vitamin, the enzyme-producing cell must have an alternative means of supporting cell growth such as synthesizing the required product of the enzyme reaction *de novo*.

Another example of the complexity of soil structure complicating soil microbial development relates to the concurrent occurrence of the enzymes and the microbes producing them. The microbes and requisite processes could be separated in time. Again, our concept of enzymes in the field must be expanded beyond that normally acquired in general microbiology or biochemistry courses. After completion of those basic science studies, enzymes may be viewed as reasonably labile components of living cells. In a soil system, enzymes synthesized by members of the soil community may become separated from the cell producing them in time. In this situation, enzymes become covalently bound to soil humic substances; that is, they could be humified. Should this occur and the binding of the enzyme to the humic acid does not obscure the active site (location on the enzyme where the substrate interacts with the enzyme to be transformed into the product), the enzyme could still be active. The physical structure of the enzyme could be altered sufficiently for a change in the kinetics of the reaction to occur. Once humified, these enzymes assume degradation susceptibilities similar to those of the humic acid; that is, weeks, months, years, decades, even centuries. Thus, the bound enzyme can survive in soil long past the lifetime of the cell that produced it; that is, they are separated in time. A specific term – "abiontic" – has been coined to describe this situation (Skujins 1967). Recall *abiotic* indicates without life whereas *biotic* refers to the living systems. An *abiontic* substance is produced by the living system, but has been separated from the microbes synthesizing it. That is, the substance is a product of the living world but has become part of the nonliving world.

These introductory observations reveal the complexity of the ecosystem in which enzymes must function in soil and the importance of assessing the chemical and physical limitations to biological activity therein. Soil enzymes are essential in assessing ecosystem function yet the complexity of the soil system complicates interpretation of data involving these catalysts. Thus, this chapter is presented with the overall objective of developing a basic understanding of the nature of soil enzymes and their role in their ecosystem.

6.1 A Philosophical Basis for the Study of Soil Enzymes

Soil enzymology has been an accepted scientific study area for about 100 years. A large body of published reports document extensive scientific effort directed at (i) the evaluation of the variation in the quantities of numerous enzymatic activities with soil and ecosystem type as well as at (ii) the optimization of the procedures for measuring these activities. These data provide a basis for developing a conceptual model of the soil interactions affecting enzymatic activity.

As a starting point for understanding the diversity of soil enzymatic activity, it can be reasonably assumed that all enzymes necessary to support growth and replication of indigenous soil populations are present in all soil samples with active microbial populations. The impact of soil particulates (e.g. adsorption), chemical properties (e.g. pH, salinity), or the presence of inhibitors (e.g. heavy metals) may preclude direct quantification or result in underestimation of the total level of a enzymatic activity present in a soil sample, but the principles of comparative biochemistry predict that all essential metabolic enzymes are produced by metabolically active soil microbes. Levels of enzyme activities detected in a soil and their utility are impacted by (i) the diversity of natural and xenobiotic organic compounds of the soil system, the chemical and physical complexity of the system, and the extreme heterogeneity of the microsite wherein the enzymes must function as well as (ii) the history of the site (e.g. growth of invasive species, conversion of native lands to agricultural production or simply changing the cropping practices).

As noted above, all enzymatic activities required to sustain microbial life logically must be present in a biologically active soil. With this basis for anticipating the diversity and function of enzymes in soil, the next logical concerns in assessing their activity become how the varying physical and chemical properties of the soil matrix control the levels of enzyme activity expressed therein, which enzyme activities are best suited as indicators of the microbial potential, and how do enzyme levels relate to overall soil function and ecosystem sustainability? These questions will be considered through the examination of current and historical reports concerning use of soil enzymes as assessors of such concerns as properties

of highly managed soils, chemically polluted soil systems, and reclamation of degraded soil ecosystems. Examples of studies relating to our general understanding of soil processes as well as practical applications to increase our understanding of soil biological responses to societal development on the productivity and sustainability of soil systems are examined. (For additional insight into these topics, see the review prepared by Burns et al. 2013.)

- *Evaluation of basic properties of enzymes in soils:* This would include determination of the source of the enzymatic activity, the kinetics of the process catalyzed, physical limitations to enzymatic activity, stability of the enzymatic activity in soil, and the variation of activity with soil and ecosystem type (e.g. Duxbury and Tate 1981; Sarkar et al. 1989; Tateno 1988; Zantua and Bremner 1975) as well as the basic reactions associated with nutrient cycling (e.g. the nitrogen, carbon, and phosphorus cycles) (e.g. Bowles et al. 2014; Turner et al. 2014). Specifically, phosphatases, sulfatases, and nitrogen oxide reductases have been used to estimate potential to catalyze essential portions of the carbon, phosphorus, sulfur, and nitrogen cycles, respectively (e.g. Häussling and Marschner 1989; Tarafdar and Jungk 1987; Tate 1984). General application of such studies is exemplified also by Stone et al. (2014) who noted that several enzymatic activities were useful for examining microbial physiological properties across several orders of magnitude of soil carbon in two soil orders (Oxisols and Inceptisols) at different elevations at six soil depths tropical forests in northeast Puerto Rico.
- *Enzymes as an indicator of soil fertility:* Soil fertility is based on a composite of soil traits, including both biological and abiotic soil properties. Examples of enzymatic activities studied as indicators of soil fertility include dehydrogenase, a variety of carbon cycle enzymes (e.g. cellulose, α-glucosidase, β-glucosidase, N-acetyl-β-D-glucosaminidase, β-xylosidase, peroxidase, phenol oxidase, and cellobiohydrolase), nitrogen cycle enzymes (e.g. N-acetyl glucosamindase) and phosphorus cycling enzymes (acid and alkaline phosphatase). Historically, considerable research effort has been expended to elucidate the relationship between these and other basic enzymatic activities and overall soil fertility (e.g. Moore and Russell 1972; Verstraete and Voets 1977). Considering the diversity of soil properties controlling enzymatic activity, contrasting conclusions regarding the utility of assessing specific soil enzymatic activities as direct indicators of soil fertility must be anticipated. For example, Moore and Russell (1972) found little linkage between dehydrogenase activity, an activity directly associated with microbial respiration, and soil fertility. Similar results were noted by Brendecke et al. (1993) with sewage sludge-amended desert soils. Verstraete and Voets (1977) noted that yields of winter wheat related positively to phosphatase activity whereas a negative relationship of this enzymatic activity to sugar beet yields was observed. Soil enzymatic activity has also been shown to reflect cropping and soil management

practices (e.g. Chander et al. 1997; Curci et al. 1997; Kramer et al. 2013; Mangalassery et al. 2015; Ross et al. 1995; Serrawittling et al. 1995).

The contrasting observations do not negate conclusions supporting the utility of enzymatic data in documenting enzyme–fertility relationships but rather they reveal the complexity of the relationships of enzyme, soil fertility, and associated soil properties. Thus, soil enzyme profiles may serve as an indicator of changes in soil structure and organic matter levels due to alteration of system management programs.

- *Enzymatic activity as an indirect measure of microbial biomass:* Considerable effort has been expended in determining the relationship between the levels of particular enzymatic activities and total microbial biomass or soil respiration. Theoretically, any metabolic activity expressed in active cells in concentrations proportional to the number of cells could be used to measure microbial biomass. This activity must be directly associated with the microbial cells and be rapidly inactivated or destroyed with cell death. A common enzymatic activity used to estimate microbial activity is dehydrogenase (e.g. Casida 1977). Dehydrogenase activity may be proportional to soil respiration but direct correlation with microbial population density is frequently not observed (Frankenberger and Dick 1983; Tabatabai 1982). Frankenberger and Dick (1983) found that alkaline phosphatase, amidase, α-glucosidase, and dehydrogenase activities significantly correlated with microbial respiration in glucose-amended soils. No correlation was measured in unamended soils. Alkaline phosphatase, amidase, and catalase activities did correlate with microbial biomass.
- *Indicator of the effects of pollutants:* General overall ecosystem stability is related to the "general health" of the soil microbial community. Disruption of soil microbial activity as shown by changes in levels of metabolic enzymes can serve as an estimate of ecosystem disruption. This relationship is clearly shown when soils are polluted with heavy metals or a variety of toxic organic compounds. In regard to the metals, the metal may combine with exposed enzymes through association with sulfhydral groups, thereby altering the tertiary structure of the protein sufficiently to reduce or destroy the enzymatic function (e.g. Cole 1977; Doelman and Haanstra 1979, 1989; Mathur and Sanderson 1980; Tyler 1981). For example, Doelman and Haanstra (1979) found increasing inhibition of soil respiration and dehydrogenase activities with increasing lead concentrations from 375 to 1500 μg $Pb\,g^{-1}$ dry soil. Similarly, Cole (1977) noted inhibition of amylase, β-glucosidase, and invertase in lead amended soils. Similarly, enzymatic activities can be reduced by inputs of toxic organic compounds. For example, Stromberger et al. (2005) noted that acid phosphatase and sulfatase were impacted more by methyl bromide than were dehydrogenases and β-glucosidase. An example of differing impacts of organic pollutants on enzymatic activity was provided by Bello et al. (2013) for soils amended with

2,4-dichlorophenol and 2,4,5-trichlorophenol. In the latter study, the amount of pollutant added to the soil, the total soil carbon content, and soil pH appeared to be major controllers of the enzymatic activities. Fluctuations of soil enzymatic activities have been shown to be useful for quantification of changes in soil biological activities due to inputs of molecules ranging from carbon dioxide (useful for evaluation of soil community responses to global climate change) (e.g. see Kandeler et al. 2006; Nie et al. 2014) to complex organic wastes, such as organic compost from sewage sludge residues, municipal solid waste, or sheep manure or manure-based biogas fermentation residues (Insam et al. 2015).

- *Enzymes as a predictor of bioremediation:* As the biological community is reestablished in damaged soils, associated enzyme activity increases. Similarly, in organic chemical-contaminated soils, increases in enzymatic activities involved in mineralization of the pollutant serve as a predictor of capacity of native populations to return the soil to previously existing conditions (e.g. Burns and Edwards 1980; Dick et al. 1988; Klein et al. 1985; Stroo and Jencks 1982). Accordingly, a variety of enzymatic activities in soil have been used to monitor metal polluted sites, such as ecosystems associated with metal smelters (e.g. Brookes 1995; Kandeler et al. 1996; Kelly and Tate 1998). Enzymatic activity utility in assessing responses of soil quality to management processes is exemplified by research of Quideau et al. (2012), using β-glucosidase, acid phosphatase, and phenol oxidase to examine the effect of amendment with several soil materials plus a range of fertilization treatments for oil sand reclamation in western Canada. Similarly β-glucosidase activity was used along with various soil physical and chemical properties to assess the microbial response to use of high lime flue gas desulfurization product for reclamation of an abandoned surface coal mine in Ohio (USA) (Chen et al. 2013).
- *Indicator of soil quality:* Although soil is a renewable resource, restoration of damaged soil sites to their optimal functional level requires decades to centuries. (See Hillel (1991) for a discussion of the decline in the quality of world soils and the resultant impact on society.) Normal soil usage for sustenance of society commonly results in declines in soil quality. Therefore, it is vitally important to manage soils in a manner that maintains or optimizes soil quality. To accomplish this end, indicators are needed to assess changes in soil quality due to normal usage as well as determination of the success of reclamation-management procedures (see Doran and Parkin (1994), Karlen et al. (1997), and Sims et al. (1997) for discussions of soil quality definitions, its assessment, and the importance of soil microbial communities in optimizing soil quality). One such tool for assessing changes in soil quality is measurement of basic soil enzymatic activity. Soil enzymes vary with management and are impacted by soil pollution. Therefore, they could be anticipated to be an indicator of variations in soil quality. For example, soil enzymatic activity has been used to assess remediation effects on industrially disturbed soils (Rowell and Florence 1993) and impacts of cropping

history on soil quality parameters (Halvorson et al. 1996; Jordan et al. 1995; Miller and Dick 1995). Similarly, revegetation of desertified lands has been assessed with a variety of enzymatic activities (dehydrogenase, catalase, α- and β-glucosidase, protease, and phosphatase) (Zhang et al. 2015).

With each of these examples, research results can be presented that demonstrate successful application of enzymological data to the achievement of the study objectives, but numerous failures have also been recorded. These conflicting results are frequently the product of a failure to fully consider the complexities of the soil environment. For a selected enzymatic activity to reflect the level of soil biological processes, the following criteria should be met.

- The enzymatic activity must be associated with living cells.
- The enzyme must be a participant in a biological reaction essential for the process of interest. (This point is particularly critical when attempting to relate enzymatic activity to soil fertility levels.)
- The activity measured must be proportional to the quantity of enzyme present. Active enzyme must not be obscured by large pools of soil-stabilized, abiontic enzyme or occurrence of spontaneous chemical transformations of the same or similar processes as are catalyzed by the enzyme.
- The enzyme activity must be essential for the process of interest (i.e. alternate synthetic pathways must not be major contributors to the conversion or the proportion of the reaction rate contributed by the alternate route must not change as the soil condition is altered.)

These observations regarding the utility of enzymes as an indication of soil biological activity as well as the considerations regarding valid interpretation of the data reveal an underlying conceptual viewpoint of the soil enzymologist. The philosophical understanding of the soil enzymologist resembles that of a biologist evaluating the enzymatic activities of plant or animal tissue more closely than it does that of a bacteriologist studying pure cultures of bacteria growing under defined conditions. For the soil enzymologist, activity of the individual microbial species is less important than the sum of all metabolic activities occurring in the soil system. Each cell, enzyme, animal, or plant tissue sample is considered merely to be a part of the larger entity, the soil. From the view of a soil microbiologist (or perhaps a better term – ecosystem microbiologist), the function of the whole is frequently more important than the intricacies of the parts. This is not to say that the identity of the individual has been trivialized. Indeed, to gain a complete understanding of the contributors to ecosystem homeostasis, as well as ecosystem potential, the potential and expressed communities of each individual life form must be elucidated and understood, but it is their interactions as a "team" rather than their "stellar performances" that are generally of utmost importance for the sustenance of the total ecosystem.

6.2 Basic Soil Enzyme Properties

At the outset, it must be noted that the basic biochemical concepts relating to enzyme function and kinetics do not differ between soil ecosystems and those grown in test tubes. That is, enzymes are proteins whose activity is characterized by parameters reflective of the interaction between the enzyme, its substrates, and the products of the reaction. Synthesis rates of *de novo* enzymatic activity are controlled by cellular metabolic processes. Some enzymes are always produced by the cells (constitutive). Others are only produced when their activities are required (inducible). A third group is intermediate between these two groups. In the latter situation, some enzymatic activities are synthesized in low levels when no inducer is present and at high levels when the activity is fully induced. Apparent variation in enzyme properties as measured in soil, which appears to be contradictory to data collected in axenic cultures or with partially purified or characterized enzyme preparations, results from the interaction of the enzyme molecules and biological cells with the variety of chemical and physical components existent in the soil microsite. Thus, the following analysis is directed at delineating the common points between *in situ* function of enzymes in soil microsites and properties of these catalysts in purified or semi-purified preparations studied in the laboratory as well as the divergence associated with understanding these entities in the laboratory and field. These observations do not indicate that unexpected results (compared to laboratory studies of more defined systems) are not encountered. Such situations present a challenge in that they can indicate that our concepts are wrong or that more research is needed to reveal the true conditions in the soil.

Soil enzymes as proteins: Enzymes are proteins that combine with their substrates in a stereospecific manner to catalyze biochemical reactions through the modification of stress on the molecular configuration around the chemical bonds at the site of catalysis, thereby lowering the activation energy of the reaction. This rather simple definition provides a basis for predicting modifications in enzymatic activity and limitations to the longevity of these activities that are imposed by soil properties.

Initial considerations relate to the proteinaceous nature of enzymes. These proteins are folded into a three-dimensional structure that optimizes interactions with the substrates for the reaction. Located within this three-dimensional structure of the protein is the enzyme's active site. The active site is the cleft in the molecule where the substrate(s) fit and are transformed into the product(s). The configuration of the active site is critical for the occurrence of the reaction. Minor changes in the structure of the protein in or around the active site can have major consequences on the rate of reaction. Thus, any soil properties that modify the protein's three-dimensional structure can alter the enzymatic reaction rate. Examples of how the structural properties of the enzyme can be affected by chemical processes within the soil components include interactions with multivalent

ions, by the ionic changes induced by variations in soil pH, changes in hydration due to desiccation, as well as by a covalent linkage to soil humic substances. The enzymatic reaction rate in soil is therefore dependent on the substrate concentrations, pH, ionic strength of the interstitial water, temperature as well as the presence or absence of a variety of enzyme inhibitors and activators. Some of these factors affect molecular configuration, whereas others involve the probability of interaction or collision of the substrate and the enzyme molecules.

Extreme disruption of the enzyme three-dimensional structure is termed denaturation. This could be likened to the changes that we observe when egg albumin is heated to high temperatures. Denaturation of enzyme proteins in soil may be induced by pH extremes, high temperatures, and interaction with a variety of polyvalent metallic anions (such as mercury, copper, and cadmium).

Enzyme vs enzymatic activity: A basic aspect of describing soil enzymes is the terminology used to designate the nature of activity measured in a soil sample. It is not unusual to hear soil enzymologists state that a particular enzyme, e.g. phosphatase, was measured in a soil sample. Generally the activity assessed is the catalysis of an organophosphate-producing inorganic phosphate under defined assay conditions. Considering that a variety of phosphatases capable of catalyzing the same reaction but with different reaction kinetics and differing optimal chemical and physical environments commonly occur in soil, for clarity, the only time that the specific name of the enzyme (i.e. phosphatase) should be used is when it is clearly demonstrated that the activity of a single enzyme has been measured especially; otherwise the specific reference should be to the enzyme activity (i.e. phosphatase activity for our example enzyme). This is particularly important for soil enzymology assessments since soils support complex biological communities functioning under highly varying optimal chemical and physical conditions. This nomenclature acknowledges the important concept that the amount of activity detected in a soil sample is the sum of contributions of all of the individual enzymes types functioning under the conditions existent in the soil sample at the time of assay. Thus, it is likely not characteristic of any specific individual enzyme.

A further qualification of the description of the soil process measured involves an indication of whether potential or actual field activity is being estimated. This point relates to enzyme kinetics parameters to be discussed later in this chapter and the specific assay conditions used to characterize the soil enzyme. Enzyme activities are generally measured in the laboratory under optimal chemical conditions with nonrate-limiting substrate levels; however, in soil, rate-limiting substrate concentrations generally occur and the enzyme must cope with such difficulties as suboptimal moisture, pH, or salinity conditions. Thus the activity measured in a test tube is the maximum rate at which the enzyme activity could be expressed, something probably rarely experienced in the soil sample. Thus, the value measured in soils is best referred to as the potential enzymatic activity.

Role of enzymes in soil: The most clearly delineated enzymatic activities in soils are those cytoplasmic activities associated with intermediary metabolism, especially energy production. Less distinct is the contribution of extracellular enzymes to the metabolism of living cells. Extracellular enzymes are secreted by soil organisms to solubolize or metabolize substrates external to the cell in order to produce nutrients needed by the microbes, to detoxify environmental substituents, or to modify the microenvironment of the living cell in a manner to improve the probability of cellular survival and ultimately replication. These enzymes are highly valuable for total ecosystem function as long as the products of their activity are available to living cells. Note that a positive benefit for ecosystem function may be credited to extracellular enzymes as long as *any* living entity benefits. Thus, the cell producing the enzyme may be at a disadvantage, but the community gains. Should these products or cells requiring the products of their activity not concurrently exist, the extracellular enzymes could be classed as having no ascribable role in the soil ecosystem. For stabilized enzymes involved in processes associated with soil nutrient cycles, a community benefit is more easily postulated. For example, phosphatase catalyzes the conversion of organic phosphates to mineral phosphate. This production of a required nutrient by a common soil enzyme clearly has a role in maintaining general soil fertility – in agricultural systems as well as in native, unmanaged ecosystems (i.e. those not receiving external phosphate sources).

Soil enzyme activity longevity: Since soil enzymes, be they within the living cell, existing free in soil interstitial water or bound to colloidal soil organic matter and clay particles, are proteins and since proteases (enzymes which hydrolyze proteins to amino acids) are ubiquitous in soil, enzymes are reasonable labile soil substituents. As indicated above, some enzymes may be stabilized in soil organic matter and therefore have a half-life of decades or even centuries (Skujins and McLaren 1969), but most enzymatic activities are reasonably short-lived. Those enzymes contained within cellular protoplasm, i.e. associated with intermediary metabolism, turn over at rates dictated by normal cellular metabolism. The change in enzyme concentration within the cell with time (dE/dt) is described by the following general equation:

$$dE / dt = dE_{synthesis} / dt - dE_{decomposition} / dt \tag{6.1}$$

For intracellular enzymes, these rates are controlled by the metabolic status of the cell. In a starving cell or even a resting cell, the decomposition rate may actually be much larger than the synthesis rate – especially for enzymes catalyzing nonessential processes – so that the quantity of a particular enzyme activity rapidly declines.

External to the cell, some enzymes may be stabilized by covalent linkage to soil humic acids. The physical attachment of the enzyme to these substances limits access to the enzyme by proteolytic enzymes, thereby reducing the potential for

proteolysis. This covalent linkage of the enzyme proteins to soil organic matter also stabilizes the enzyme structure in a manner that reduces the potential for denaturation of the protein. For enzyme activities that are a combination of endo- and extracellular activities, the apparent change in total activity in soil is modulated by the quantity of stabilized enzyme. That is, total enzyme activity (E_T) is described as follows:

$$E_T = E_{stabilized} + E_{cellular} \tag{6.2}$$

where the quantity of cellular enzyme and its turnover rate are described by Eq. (6.1). The practical result of this relationship is that in systems containing large quantities of a particular stabilized enzyme, such as phosphatase, the changes in newly synthesized enzyme can be obscured by the quantity of stabilized enzyme. In these situations, quantities of enzyme measured in soil samples do not reflect variation in activity of the living biomass.

Stabilization of soil enzymes: Since a significant portion of the enzymatic activity detected in soil samples may be the result of enzymes covalently bound to colloidal soil organic matter or associated with clay particles, consideration of the nature of these stabilized proteins and the processes leading to their long-lived state is meaningful. There are two major fates of enzymes lost from cells due to cell lysis or to active secretion of the proteins into the extracellular milieu: the proteins may be decomposed or they may be stabilized through linkage to soil humic materials or adsorption to clay minerals.

Enzymes whose native environment is the cell cytoplasm, the cell membrane, or the periplasm generally find the environment external to the cell harsh. These proteins are vulnerable to suboptimal pH, unfavorable ionic strength of soil interstitial water as well as the presence of denaturing agents, such as heavy metals. Sensitivity to the external milieu varies with the nature of the enzyme protein. For example, Frankenberger and Bingham (1982) found a differential sensitivity of a variety of intra- and extracellular enzyme activities to soil salinity. Dehydrogenase activity was limited by salinity whereas a variety of hydrolases were more resistant. The authors concluded that osmotic desiccation of the microbial cells may have resulted in release of the intracellular enzymes into the soil interstitial water and their subsequent proteolysis.

Adverse conditions tend to disrupt the protein structure in a manner to increase susceptibility to proteolytic activity. Within the cell, proteins may be protected by physical separation of the proteins and proteases as well as existence of the protein in a structure that limits or perhaps even precludes proteolytic attack. For example, should the terminal amino acid residues be protected by being embedded within the protein molecule, C- or N-terminal-specific proteases would not be able to initiate hydrolysis of the protein. This protection could be lost once the protein normally

contained in the cytoplasm enters the soil environment. The conditions external to the cell encourage unraveling of the normally intracellular resident enzyme so that proteases may more easily attach to and cleave the protein.

Those enzymes that are synthesized by the cell and secreted for function external to the cell wall or membrane (extracellular enzymes) generally have a tertiary structure that is more strongly held together by covalent, hydrogen bonding, and ionic linkages than occurs with intracellular enzymes. Thus, extracellular proteins are more resistant to denaturation and proteolytic activity than are enzymes whose prime site of function is the protected core of the cellular protoplasm.

As indicated above, enzymes can be stabilized through ionic, hydrogen, or covalent bonding to soil humic materials. In situations where the linkages is through ionic or hydrogen bonds, the enzymes can be easily removed from the soil by washing in buffer suspensions. For example, peroxidases can frequently be easily extracted from soil suspensions (Bartha and Bordeleau 1969). Few enzyme molecules are extractable from soil with buffer solutions, so it is logical to conclude that this type of association between enzyme proteins and soil organic matter is somewhat uncommon. Similarly, although there are numerous reports of separation of soil enzyme activities from mineral matter (e.g. Ladd 1972; Perez-Mateos et al. 1988), these purified enzyme preparations still contain humic substances associated with the proteins. Gosewinkel and Broadbent (1986) did separate about 3% of the phosphatase activity from a field soil with electron-donating substances, suggesting a disruption of the enzyme-humic acid covalent linkage. The proteins may be bound to humic acids through quinones by nucleophilic substitution, to sulfhydral groups, or to terminal and ε-amino acids. In these situations, enzymatic activity of the proteins is maintained if the protein configuration is maintained and the bonding does not obscure the active site. Should either of the conditions not be met, then the enzyme would enter the class of humic acid-bound proteins rather than being considered to be a soil-stabilized enzyme.

The quantity and type of enzymatic activities stabilized in particular soil samples may be distinctive for the individual soil site, such that their analysis could be of value for forensic investigations. Thornton et al. (1975) found that phosphatase, arylsulfatase, urease, invertase, and trypsin activities in three soil series were sufficiently characteristic of the soils that soil samples collected within close proximity could be distinguished by their enzymatic patterns. Michaelis constants of the enzymes could be used to characterize the soil samples.

6.3 Principles of Enzyme Assays

To quantify a specific enzymatic activity in soil samples, knowledge of a variety of properties of the enzyme is essential, even if the activity of interest has been studied previously. In the latter situation, the enzyme assay must still be optimized for

the particular soil of interest and perhaps modified to increase the probability of achievement of the experimental objectives. Contrasting goals for a study may be (i) to quantify total activity of a particular enzyme activity present in a soil sample or (ii) to elucidate the amount of that activity expressed *in situ*. These values may differ. For example, the enzyme activity may be measured in a buffer with a pH that is optimal for a particular enzymatic activity. This may allow determination of the highest rate of substrate transformation by an enzyme in the soil sample. Unfortunately few enzymes are functioning in soil at their optimal pH. Thus, activity values detected in buffered assays may show little relationship to real field catalysis rates. To best estimate field activity rates, the assay should approximate the conditions where the enzyme normally functions. Note that even if the assays are conducted at the prevailing soil pH, the level of activity detected still only reflects potential activity. This fact results again from the ideal conditions provided during the quantification of the enzymatic activity. Other variables generally optimized are substrate concentration, interaction of the substrate and enzyme (the assay mixtures are generally mixed while the enzyme is being quantified to maximize interaction between the enzyme and its products), and temperature.

To provide a reasonable and reproducible estimate of activity of an enzyme *in situ* in the soil sample, several properties of the enzyme and the reaction catalyzed must be understood in detail (see Tabatabai 1982 for a more detailed discussion of this point).

- The reaction catalyzed should be understood in stoichiometric detail. This includes knowledge of the reactants, the products as well as an understanding of the chemical and physical properties of each of these chemicals. These properties may be useful for quantification of the reactants and products of the enzymatic quantification. For example, a strong ultraviolate or visible light adsorption maximum could be exploited for a quick assay of product formation or substrate disappearance.

  Competing fates of the product, or of the substrate, also must be understood. These can lead to over- or underestimation of the actual enzymatic activity. For example, sorption of the substrate to soil particles (such as clay) could result in production of data that suggest greater transformation of the substrate than actually occurs (if substrate disappearance serves as the indicator of the reaction). Alternatively, if product appearance is quantified, utilization of the product in another reaction could result in underestimation of the enzymatic activity.
- The requirement for chemicals besides the substrates for optimal enzyme reaction should be understood. This would include any necessary metal requirements for the proteins as well as electron acceptors. This is less of a problem for assay of enzymes in a crude milieu such as soil, but becomes a greater difficulty as the enzyme activity is "purified."

- The kinetics of the reaction in relationship to substrate concentration must be elucidated. Enzyme assays are generally conducted with enzyme rate saturating concentrations of the substrate. This procedure results from the necessity of having the assay proportional to the quantity of enzyme present and not limited by the substrate levels. An exception to this is encountered when saturation levels of the substrate are inhibitory to the enzyme. In this situation the V_{max} of the reaction can be used to estimate the quantity of the enzyme present. (See Section 6.4.)
- Even though the experimental objective may be to quantify the enzyme activity under field conditions, the optimum pH, temperature, and ionic strength of the enzyme activity should be understood. This is particularly important when a variety of soil types are to be assayed. Similar levels of enzyme protein may be present in each soil, yet the expressed activity could vary significantly due to occurrence of suboptimal chemical or physical conditions in either the soil or the reaction mixture.
- A suitable means of monitoring changes in the substrate or product must be developed. This is particularly important in the soil system since the altered conditions of the soil in the assay vessel could cause synthesis of new enzymatic activity or even, in rare situations, result in a decline in enzyme levels. Generally, it is more expeditious to measure changes in product levels since it is generally more accurate to quantify increases in level of a compound from zero than to measure slight changes in a substrate that is present in high concentrations.

Methodological difficulties of assessing soil enzymatic activity: Even after developing an apparently ideal assay for the enzyme activity of interest, application of the procedure to soil samples may be precluded or special considerations may be necessary in interpreting the results of the procedure. As has been stressed above, these difficulties arise primarily because of the complexity of the soil system and the reactions occurring therein. Four specific problems will be used to illustrate these difficulties: (i) biologically versus chemically catalyzed processes, (ii) complications due to microbial growth or enzyme synthesis during the assay time, (iii) stability of the enzyme products in the reaction mixture, and (iv) impact of sample treatment prior to enzyme assay.

Biological vs chemical catalyzed processes: Within the reasonably defined confines of a microbial culture vessel, it is somewhat easy to assign cause and effect to a particular process, i.e. biological and chemical reactions are usually easily distinguished. In soil, because of the potential for catalysis of organic transformations by clay particles, abiontic enzymes, as well as occurrence of spontaneous chemical reactions, such distinctions are much more difficult. It is not unusual for a chemical conversion to be catalyzed both biologically and chemically within the same soil sample, and perhaps even within the individual microsite. Although it is catalyzed by intracellular enzymes, denitrification provides an excellent example of the

overlap between biologically catalyzed and spontaneous chemical reactions at acidic pH values. Denitrification is predominantly the result of enzymological catalysis of the reduction of nitrogen oxides in agricultural soils with mildly acidic (5.5–7.0) pH levels. As the soil pH declines, nitrogen oxides may be denitrified through spontaneous chemical reactions. At the acid pH extremes (<3.5), although it has been shown that biological denitrification can occur (Muller et al. 1980), it is generally assumed that chemical reactions predominate. At the intermediate pH ranges, both chemical and biological reactions can occur.

The question thus becomes, "How can biological and chemical processes be distinguished in soil?" Three reaction properties that may separate abiotic or biotic contributions to a soil process are the impact of sterilization of the soil on the reaction, heat stability of the enzymes, and reaction kinetics.

The most commonly used procedure for distinguishing biological processes from spontaneous chemical reactions is to assess activity in sterile soil controls. Biological activity may be destroyed by steam sterilization (121 °C, 15 psi), but a variety of chemical sterilants as well as radiation sterilization have been used. The assumption underlying these methods is that only the biological aspects of the soil are altered by the sterilization procedure and that the chemical processes continue at unaltered rates in the treated soil.

Ideally, an all-or-none effect on the reaction rate by sterilization is observed. Unfortunately, frequently, such easily interpreted data are not produced. The reaction rate may be reduced by sterilization but not precluded. Contributions to the intermediate effect include the possibility that the soil samples may not have been rendered sterile by the sterilization procedure, the process may actually be occurring as a result of chemical processes, at least in part, or sterilization of the soil created conditions where the chemical reaction could occur when it would not necessarily be anticipated to occur in the native soil sample. Radiation-sterilized soil samples are particularly vulnerable to the latter problem. Radiation sterilization creates free radicals in the native soil organic matter. For transformations such as polymerization of aromatic ring-containing compounds, formation of free radicals increases the probability of incorporation of aromatic ring-containing chemicals, for example, into native soil organic matter as well as polymerization of the test compounds. Alternatively, the assumed sterility of the soil may not have been achieved or maintained during the analysis period. A sterility control must be conducted. Samples of the treated soil should be examined for surviving microbial populations, or any that developed subsequent to sterilization but prior to use in the experiments, by culturing soil samples in a variety of media. Testing for microbial growth in a single growth medium does not exclude the existence of viable microbes unable to grow in the test medium but capable of growth in an alternate medium.

Heat stability of the catalysts can also be used as a measure of enzyme-catalyzed reactions. Proteins are denatured quickly (within minutes) by temperatures above

55 °C. Thus, heating of a soil sample at 55 °C for approximately 30 minutes should result in destruction of an enzyme-catalyzed reaction. Again, interpretation of soils data must be tempered with the realization that proteins stabilized in native soil organic matter may become reasonably heat resistant. Thus, a portion of the enzyme activity may survive the heat shock.

A further consideration in differentiating chemical and biological soil processes relates to reaction kinetics. As shown below, saturation kinetics are observed with enzyme-catalyzed processes. As the substrate concentration is increased in the reaction mixture, a level is reached where no further increase in reaction rate is detected (see Figure 6.1). The reaction becomes proportional to the quantity of catalyst present rather than the substrate concentration. In contrast, increased concentrations of reactants in a chemically catalyzed mixture generally leads to increased product yields until another rate limitation is imposed, such as substrate solubility. Therefore, an evaluation of the reaction kinetics can frequently be used as an indicator of biological vs chemically catalyzed reactions.

Complications due to microbial growth or enzyme synthesis: Another methodological difficulty is associated with the assay procedure itself. Again, with a defined mixture of enzyme substrates, cofactors, and buffer, it is reasonably easy to keep

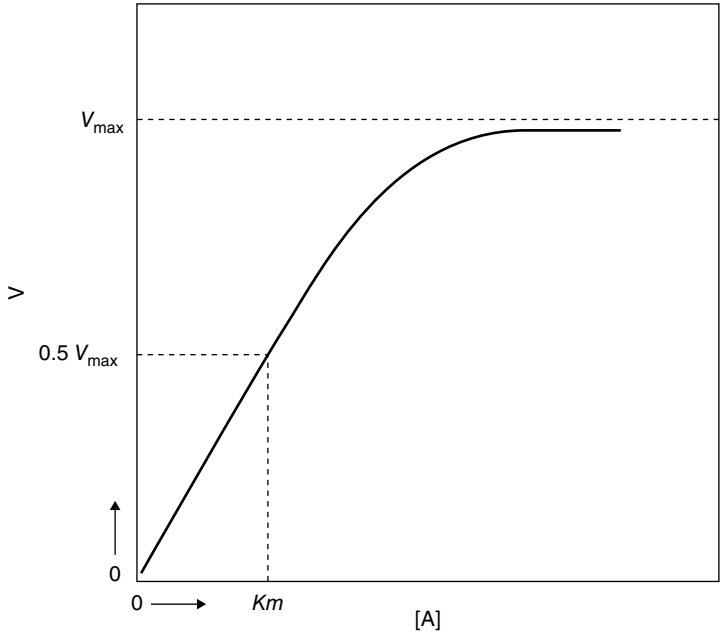

Figure 6.1 Generalized rate curve for an enzyme-catalyzed reaction where V_{max} is the maximum reaction rate, v is the reaction rate, and [A] is a substrate for the enzyme.

the principles (the enzyme concentration and substrates, including any requisite cofactors) at constant or saturating levels throughout the assay period. This is frequently difficult, if not impossible, in evaluating soil enzyme activities. Soil samples are a complex mixture of substances that may allow replication of enzyme-producing cells as well as *de novo* enzyme synthesis by preexisting microbial populations. Conditions are generally ideal for growth of the enzyme-producing microbial cells or synthesis of nascent enzyme during enzyme assays.

Since the objective with any assay procedure is to determine the quantity of enzyme activity present in the soil sample prior to assay, not that produced during the quantification procedure, enzyme synthesis must be prevented, or at least minimized. For assays of a few minutes duration, enzyme synthesis is not a significant problem, but some procedures require incubation times of several hours for adequate product to be synthesized for accurate quantification. For example, phosphatase and some protease activity measurements require 4–6 hours or longer. A variety of microbial growth or protein synthesis inhibitors may be incorporated into the enzyme assay mixture. Inhibitors of microbial growth commonly used in soil enzyme assays are toluene, γ-irradiation, and antibiotics. Toluene (10–25% [volume/volume] concentrations) is the oldest, and is still relatively commonly used. The inhibitory mechanism of toluene is primarily the dissolution of cell membranes. Therefore toluene inhibition of cell growth is most useful for assessment of extracellular enzyme activity and for those activities where disruption of the cell membrane does not affect the activity. It has been used for phosphatase and invertase activities. Dehydrogenase activity – a nondescript enzyme activity that is associated with intact cell membranes – is inhibited by toluene inhibition.

In contrast, irradiation of cells with 5–10 Mev electron bean, hard x-rays or γ-rays is useful for assay of intracellular as well as extracellular enzymatic activity. Of consideration in using irradiation is the differential sensitivity of soil biomass and the potential to effect spontaneous chemical reactions. (See Skujins (1967) for a more complete discussion of radiation sterilization applicability to enzymatic assays.)

Antibiotics have also been used to prevent *de novo* protein synthesis during soil enzyme and respiration measurements. Considerations in interpretation of data derived from use of antibiotic inhibitors relate to the differential sensitivity of soil biomass (bacteriocides versus fungicides), inactivation of the antibiotic due to adsorption to soil components, and biodegradation susceptibility of the inhibitors. The degree of inhibition and specificity of the antibiotic should be examined for each soil type to be used in the individual study.

Stability of enzyme products in the assay mixture: Further methodological complication to be considered in developing enzyme assays involves the fate of the reaction product. As indicated above, the most sensitive enzyme assays involve quantification of the reaction product. With defined systems, it is relatively easy to chose a substrate or modified substrate that yields products that are not further

metabolized within the reaction mixture. This goal is frequently not possible with an ill-defined soil sample. The product of the enzyme activity may be an ideal substrate for other microbes or enzymes existent in the soil mixture. For example, it is common to measure carbohydrases by quantifying the monosaccharide production rate, but in soil, monosaccharides are easily degraded, and frequently short-lived. Thus, a control for such assays must involve a determination of the longevity of the monosaccharide yielded by the enzyme.

Impact of sample treatment prior to enzyme activity assessment: The final methodological problem to be analyzed herein relates to soil sample storage techniques. Few enzymatic activities can be measured immediately upon selection of representative soil samples. Soil samples must be collected, transported to the laboratory, generally stored for some period of time, and then assayed. Procedural details that may impact the accuracy of the enzyme assay include conditions during sample storage (temperature [−21 or 4 °C, or room temperature] or moisture [field moisture or air dried]), sterilization procedure for the assay, reaction conditions (pH, temperature, etc.), and static versus agitated incubation of the reaction vessel. Controls must be used to estimate changes in field enzyme levels by each of these procedural variations.

Along with the controls indicated above, any enzyme assay procedure must include a no soil control (to demonstrate stability of the reaction mixture and with colorimetric assays, to indicate background absorbance of the reaction mixture) plus a no substrate control. With the chemical complexity of soils, the possibility exists for compounds to occur in the soil sample that mimic the product to be assayed or sufficient substrate may preexist in the soil sample to allow the synthesis of the reaction product in the absence of amended substrate.

6.4 Enzyme Kinetics

Evaluation of the impact of substrate concentration on rate of product formation of an enzyme catalyzed reaction (i.e. measurement of the kinetics of the enzymatic catalyzed reaction) is a valuable means for elucidating such enzyme and ecosystem properties as the following:

- the properties of the soil microsite in which the enzyme is functioning
- the number of enzymes catalyzing the reaction of interest that are present in the soil sample
- the total amount of enzyme in a soil sample, and the impact of soil properties on expression of the enzyme activity.

This type of assessment generally involves determination of process reaction rates with increasing substrate concentration, under otherwise constant assay conditions. Rates of soil biological processes are generally either zero-order (the reaction

rate is constant and independent of the substrate concentration) or first-order (the rate is proportional to the substrate concentration). Modification of incubation temperature, pH, ionic strength of the incubation solution, plus alteration in concentration of minor elements or cofactors required for the reaction may have a significant impact on the reaction rates.

Zero-order biological processes in soil: The disappearance of substrate (dS/dt) for zero-order reactions is described by the following simple equation:

$$dS / dt = k \tag{6.3}$$

The constant k is independent of substrate concentration and thus is proportional to total enzyme. Although first-order kinetics are more commonly recorded with soil enzyme-catalyzed reactions, zero-order kinetics are frequently encountered, especially in situations where the substrate concentrations used in the assay procedure are meaningfully larger than would normally exist in the soil site.

Observations of zero-order kinetics for soil processes commonly reflect availability of the rate-limiting substrate for the transformation rather than a basic property of the enzyme protein. Note that with the first-order reaction curve (as depicted in Figure 6.1), in the presence of high substrate concentrations the reaction rate is independent of increased substrate amendment. That is, the enzyme present in the reaction mixture is saturated and no further increase in the reaction rate due to increases in the amount of substrate in the mixture is possible. Any enzyme reaction may thus appear to be zero-order if rate-saturating quantities of substrate (i) are used in the reaction mixture to quantify the enzymatic activity or (ii) are already existent in the soil sample due to the presence of high indigenous levels of reactants. (This saturation velocity will not be apparent with enzymes that are inhibited by high substrate concentrations. With inhibition of the enzyme, the reaction rate actually declines with increasing substrate availability is elevated too high.) Thus, it is highly probable that some reactions considered to be zero-order in native soil samples are in reality first-order processes functioning at saturating levels of substrate.

Alternatively, zero-order kinetics are also observed when access to the enzyme by the substrate is limited. In this situation, the reaction rate is controlled by the rate of encounter of the enzyme and protein. This situation occurs when the substrate must diffuse to the site where the enzyme is located or where the substrate availability is controlled by its limited water solubility. In the latter situation, the effective concentration of the substrate is controlled by its dissolution rate.

First-order processes in soil: In contrast, rates of first-order processes are substrate dependent. The most commonly used mathematical representation for first-order reactions is the Michaelis–Menten equation. This relationship and some common transformations are presented in Figure 6.2. Other mathematical models have been developed for describing first-order reaction kinetics, but most soil enzymology is still based on the Michaelis–Menten equation. Note that the

$$A + E \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \times \underset{K_4}{\overset{K_3}{\rightleftharpoons}} P + E$$

Figure 6.2 Michaelis–Menton equation and some conversions which are useful in calculating kinetic constants.

Michaelis - Menten Relationship:

$$v = \frac{V_{max}[A]}{K_m + [A]}$$

where $V_{max} = K_3 E_t$

$$K_m = \frac{K_2 + K_3}{K_1}$$

Lineweaver Burk Transformation:

$$1/v = 1/V_{max} + (K_m/V_{max})(1/[A])$$

plot $1/v$ vs $1/[A]$

slope = K_m/V_{max} ; intercept = $1/V_{max}$

Hanes - Wolf Transformation:

$$[A]/v = K_m/V_{max} + (1/V_{max})[A]$$

Eadie - Hofstee Transformation:

$$v = V_{max} - K_m v/[A]$$

Michaelis constant (K_m) is proportional to three rate constants in the general enzyme reaction depicted and the maximum reaction velocity (V_{max}) is proportional to the total quantity of enzyme in the reaction mixture. (Those unfamiliar with the derivation of the basic Michaelis–Menten relationship should consult an elementary biochemistry text.)

Plotting the reaction rate vs substrate concentration yields a hyperbolic curve (Figure 6.1). Note that the Michaelis constant is that concentration of substrate that allows the reaction to proceed at one half the maximum velocity (0.5 $V_{max} = K_m$). whereas 0.1 K_m and 10 K_m are approximately equivalent to 0.1 and 0.9 V_{max}.

It is difficult to derive rate constant values from a hyperbola. The maximum velocity is an asymptote and it is reasonably difficult to distinguish with precision the substrate concentration yielding half the maximum velocity for determination of the Michaelis constant. Therefore, a variety of mathematical transformations have been derived for the reaction which result in straight lines when plotted (Figure 6.2). With the Lineweaver Burk Transformation, the slope of the line is

equal to the Michaelis constant divided by the maximum velocity (K_m/V_{max}) whereas the x-intercept is the reciprocal of the maximum velocity. These values can thus be easily determined by linear regression of the reciprocals of the reaction velocity and substrate concentration data.

Compromises associated with determination of Michaelis constants in whole soils: Michaelis constants derived from study of whole soils rather than purified extracts in defined mixtures are best termed apparent Michaelis constants (K_{app}). This conclusion is based on the following enzymological principles. Michaelis constants are a parameter characteristic of purified enzymes. The reaction kinetics are determined using carefully defined conditions. Therefore, values derived for enzyme activities assessed in suspensions of soil particulates may not reflect the same properties that would be derived from study of the same enzyme molecules exclusive of the undefined soil matrix. Difficulties in interpreting and assigning Michaelis constants to soil enzymatic activities result from the following.

- The existence of multiple enzymes capable of catalyzing the reaction in the soil system.
- Competing reactions for the substrate (resulting in a lower concentration of substrate available to the enzyme than assumed by the experimenter).
- The existence of a rate-limiting step before the substrate reaches the enzyme of interest, which may include the enzymatic activity facilitating transport of the substrate into the cell where catalysis can occur or diffusion limitations of the substrate into the microsite of enzyme activity.

Additionally, abiotic difficulties in assessing and interpreting Michaelis constant data include complications associated with substrate adsorption to clay particles (whereby desorption of the bound substrate becomes the factor controlling enzyme substrate encounters). Suboptimal conditions for the occurrence of the reaction (e.g. presence of inhibitors, unfavorable pH, or salinity levels) may also cause inaccuracies in assessing Michaelis constants of soil enzymes.

The importance of site variation in substrate concentration on enzyme reaction rates can be seen in a classical study conducted by Tabatabai and Bremner (1971). The arylsulfatase and phosphatase activities in a variety of Iowa surface soils were assessed. These workers found that the Michaelis constant varied with soil type (as would be expected) as well as with incubation procedure. Continuous mixing of the soil samples resulted in lower Michaelis constants than were detected in static samples. The elevated Michaelis constant in the static samples reflected the reduced availability of the substrates to the enzyme. Soil particles containing the enzyme activities of interest settled to the base of the test tube during the incubation period in the static soil samples. The reaction rate was dependent upon the quantity of substrate present therefore in the base of the tube and not the total concentration in the reaction vessel. Furthermore, the quantity of substrate in the

settled soil was controlled by diffusion, a slow process. Once the localized substrate in the soil mass was exhausted, its restoration was slow. Thus, the Michaelis constants derived from the static systems were a product of the assay procedure and not necessarily related to the actual enzyme capability of the soil samples.

Application of K_m values to detection of occurrences of isoenzymes in soil: Apparent Michaelis constants can be used to demonstrate multiple enzyme forms in soil. For example, Nannipieri et al. (1980) measured a variety of enzyme activities in soil and plotted the results using Eadie–Scatchard plots. Generic examples of the types of data from this analytical procedure are shown in Figure 6.3. With a single enzyme, a linear relationship is observed (Figure 6.3a), whereas with two distinct enzymes a break in the kinetics curve is detected. The data actually appear to be nonlinear, but in reality fit two straight lines (Figure 6.3b). In the situation for activity resulting from two enzymes, the reaction rate is described by the following equation:

$$v = \left(V_{max1} S / K_{m1} + S\right) + V_{max2} S / K_{m2} + S \tag{6.4}$$

As the number of different types of enzyme proteins increases, clearly the complexity of the reaction increases. The procedure cannot be used for all combinations of enzymes. Problems are encountered with isoenzymes having highly divergent kinetics constants (Figure 6.3c). The rate curve for an enzyme with a high maximum velocity and low apparent Michaelis constant would totally overshadow the curve descriptive of an enzyme with a low maximum velocity and a high apparent Michaelis constant. In this situation, the reaction would appear to result from the activity of a single enzyme. This is an excellent means of analyzing multiple forms of enzymes in soil under the simple conditions described, but frequently the data are too complicated for such an analysis.

6.5 Distribution of Enzymes in Soil Organic Components

Experimental design for study of soil enzymological activities and interpretation of data derived from such studies must be based on a thorough understanding of the complexities of distribution of the enzyme proteins within the soil matrix. As indicated above, enzyme reaction kinetics are controlled by the degree of exposure to a variety of inhibitors and limiting chemical conditions in soil. Differential protection is derived from containment in cellular structures, suspension in soil interstitial water or linkage to soil colloids. A conceptual model of enzyme pools in soil and the movement of these materials between the various organic matter pools is presented in Figure 6.4. Central to the concept of enzyme function in soil

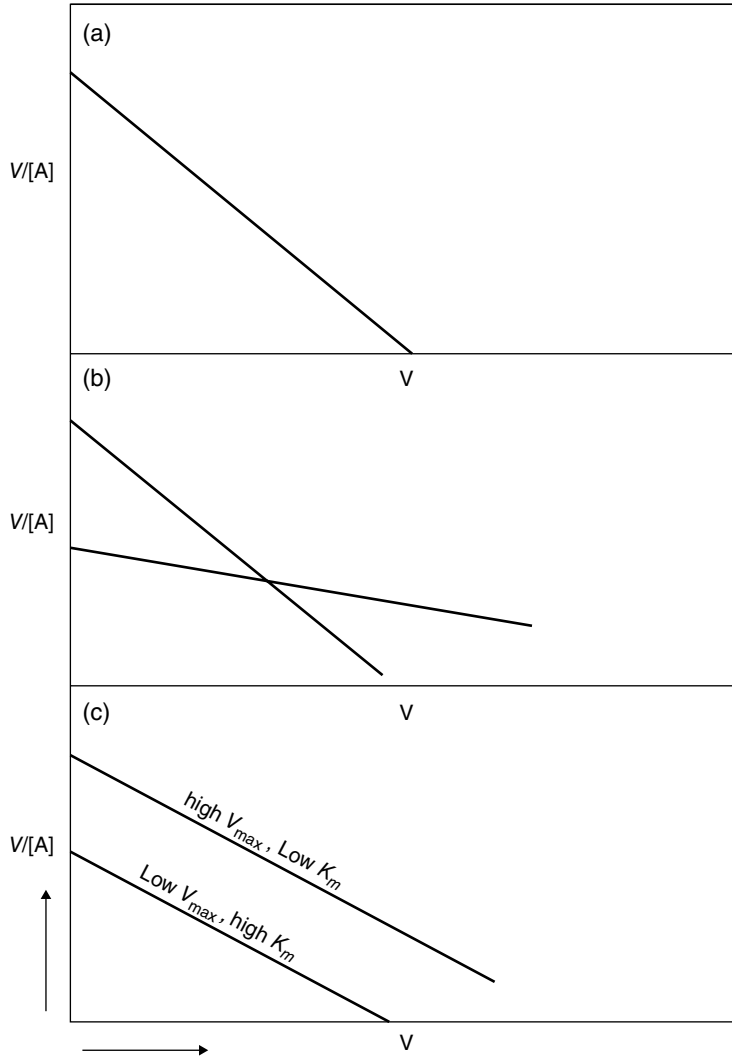

Figure 6.3 Use of Eadie–Scatchard plots to distinguish multiple forms of enzymes in soil. (a) Example of plot of data resulting from activity of a single enzyme; (b) plots demonstrating occurrence of two distinct enzymatic activities; (c) situation where multiple forms of an enzymatic activity are obscured by divergent kinetics of the two isoenzymes.

is the living cell. From a teleological viewpoint, it is for the function of the living cells that enzymes are initially synthesized. Thus, the cell must occupy the central position of the model. Two classes of enzyme molecules are linked to the living cell (for this consideration, the classic definition of a living cell will be used, i.e. a

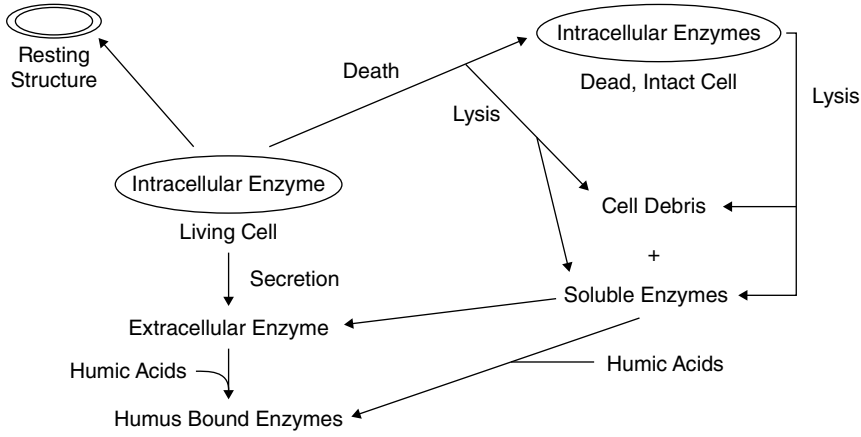

Figure 6.4 Conceptual model of the distribution of enzymatic activity within major soil enzyme reservoirs.

living cell is one that is capable of reproduction). The cell contains intracellular enzymes and is the primary source of extracellular enzymes.

Intracellular enzyme activities are those found in living microbial, plant, and animal cells. In most studies of soil enzymes, those activities associated with animal and plant cells are of less significance than those of fungal, actinomycete, and bacterial cells simply because of the mass of each cell type and their contribution to total soil respiratory and metabolic activity. Recall that in preparation of soil samples for analysis, recognizable plant material is generally removed. Most intracellular enzymes are involved in the various aspects of cellular metabolism, e.g. glycolysis, Krebbs cycle, etc. These enzymes cannot function outside the cell due to cofactor requirements and sensitivity to variation in pH, redox potential, heavy metals and other inhibitory physical conditions. For example, a variety of polyphenyl oxidases contain ferrous iron in their active center. Exposure to molecular oxygen results in oxidation of the ferrous ion to ferric ion and inactivation of the enzyme. Some intracellular enzymes are capable of maintaining their activity upon cell lysis (e.g. some proteases), but generally intracellular enzymes are short-lived outside the protective environment of the cell.

Other enzymes that are directly associated with respiring microbial cells and therefore are not classed as extracellular enzymes *per se* are periplasmic (the enzymatic activities of gram-negative bacteria and enzymes attached to the outer surface of viable cells). The activity of the latter enzymes extends into the ambient medium. In culture, periplasmic activities are distinguished from cytoplasmic enzymes by conversion of the cell to spheroplasts. This determination is clearly difficult to accomplish with soil samples, but it can be concluded that enzymes

that exist in the periplasmic space in culture would also exist in soil. Examples of this class of enzymes include alkaline phosphatase and penicillinase. Leakage of periplasmic enzymes into the external milieu would results in their being classified as extracellular enzymes.

Enzymes attached to the outer surface of viable cells are usually defined as extracellular since their activity is directed toward external functions. These enzyme activities are exemplified by a variety of bacterial polysaccharidases, but enzymes embedded in the extracellular gum of plant roots (mucigel) may also be included in this category. These enzymes catalyze the conversion of molecules whose structure precludes transport through the cell membrane into the cell into small permeable molecules.

True extracellular enzymes are those enzymes that are secreted by living cells during normal cell growth and division. These activities are found soluble in soil interstitial water. Most extracellular enzymes have low molecular weights (20 000–40 000) and are produced abundantly in soil by gram-positive bacteria, fungi, and plant roots. An easy means of differentiating extracellular enzymes is by their function. If their substrates cannot enter the cell, then it may be concluded *a priori* that the enzymes catalyzing their hydrolysis must function external to the cell membrane. Examples of such enzymes include those responsible for hydrolysis of high molecular weight or water-insoluble polymers (cellulose, hemicellulose, etc.), those involved in destruction of toxins, and those associated with dissolution of host tissue prior to invasion by a pathogen.

Other pools for activities normally classed as being intracellular are those enzymes contained in nonproliferating cells (fungal spores, protozoan cysts, plant seeds, bacterial endospores), attached to or contained within entire dead cells, and those linked to cell debris. Enzymes of nonproliferating cells are generally surrounded by thick, biodegradation-resistant walls. Thus, these activities are not detected in enzyme assays as commonly conducted unless a procedure is included that disrupts the cell structure. This task can be accomplished through sonic oscillation. Cell debris-associated enzymes are those linked to cell membranes, cell walls, plus internal structures of eukaryotic cells. These structures are rapidly decomposed, but the continuing turnover of microbial biomass assures a constant, low level of enzymes existent in this class. These group of enzymatic activities mostly includes hydrolytic activities. Longevity of enzymes in this grouping is dependent upon the degree of protection afforded by and the life-time of the cellular structure in which the enzyme is contained (cell membrane or wall) as well as the sensitivity of the enzyme to the soil environment.

Enzymes that have become stabilized in humic matter and associated with clays (abiontic enzymes) are more problematic in developing enzymes study protocols and in interpretation of experimental results than any of the enzyme pools outlined above. Synthesis of the former enzymes was induced in the cells to meet an

existing need but the enzyme activities were uncoupled from the soil biomass through association with abiotic soil components. Thus, although these enzymatic activities may be significant contributors to total activity detected in a soil sample, their presence in the soil sample may not relate to actual biological processes occurring therein. Soil-stabilized enzyme activities are a major contributor to weak or negative correlation of enzymatic activity with soil biogeochemical processes. They provide a background from which it may be difficult, if not impossible, to distinguish the modulations in cell-associated enzymatic activity. The stability of extracellular enzymes relates primarily to the longevity of humic polymers and any association with cellular function may be fortuitous. Benefit to soil biomass from these activities results entirely from the potential for enzymatic catalyzed products to diffuse to active cells.

An example of a practical implication of this dynamic distribution of soil enzymes among biotic and abiontic soil fractions is the interpretation of data derived from the analysis of air dried soils. Drying of the soil may result in redistribution of enzyme proteins among these intracellular, extracellular, and abiontic pools. Desiccation may result in death of some viable cells, lysis of viable cells and whole dead cells, as well as disruption of soil aggregates where humic or clay-associated enzymatic activity may have been occluded and therefore not able to be an active participant in soil biochemical activities. Thus, although an enzymatic activity level can be measured in air-dried soils, extrapolation of the results to actual field conditions may be difficult, if not impossible. In reality, drying of the soils creates a new ecosystem with some relationship to the native site from which the soil was collected but with new and perhaps unique properties resulting from the altered physical state of the soil.

6.6 Ecology of Extracellular Enzymes

If we may take the liberty of personifying the soil microbe, we may note that the microorganism residing in soil is faced with a major dilemma. Considerable potential energy resources that are not water soluble exist in the soil. These cannot be consumed by microbes until they are converted to a form that is moved to the microbial cell and readily transported across the cell membrane. A survival advantage is derived by any cell capable of using these substances as a carbon and energy source. The difficulty encountered is that extracellular enzymes must be used in transforming the external energy sources into units that can be consumed. These enzymes are an inefficient but essential means of procuring this energy since the probability of their function in soil and the subsequent recovery of the product of their action by the enzyme-producing cell is limited.

As indicated above, soil is an unfavorable environment for any enzyme. Even if the protein is not denatured, adsorbed, or inactivated, it may not encounter its substrate because of the heterogeneity of soil and the tendency of soil organic components to be occluded by soil mineral particulates. Furthermore, if the substrate and enzyme are found in the same microsite, the substrate concentrations may not be sufficient for optimal enzyme activity. Even if the enzyme encounters substrate in sufficient quantities to produce beneficial levels of the product, the transformation may be hindered by soil physical or chemical conditions. For example, enzyme reactions occur in a water matrix. Drying of the soil may result in too little moisture for continued enzyme activity. Even if all impediments to expression of an extracellular enzyme activity are avoided, one final difficulty must be overcome before the cell which originally synthesized the enzyme benefits from expenditure of energy resources to produce the extracellular enzyme protein. The product of the reaction must reach the cell. Since soil is a three-dimensional entity, the water-soluble product of extracellular enzyme catalysis may diffuse or be leached in paths that lead it away from the "hungry" cell. The product of the extracellular enzyme may also be consumed by cells other than the extracellular enzyme-producing cell.

A variety of adaptations increase the probability of success of extracellular enzymes. Synthesis of the extracellular enzyme may be dependent upon the presence of the substrate in the vicinity of the cell. This phenomenon reduces the probability of synthesis and excretion of enzymes with no possibility of energy gain. One means for this adaptation to be effective is for the cell to continuously synthesize a low level of the enzyme. Full enzyme production occurs only when the enzyme's substrate is present in the region of the cell. When the extracellular enzyme synthesized during the noninduced period encounters its substrate, a product is yielded that induces full enzyme synthesis. The reduced enzyme production in the absence of the enzyme substrate reduces energy expenditure and is of survival advantage to the microbe. This mechanism would only be advantageous to the growing cell in sites where there is a reasonable probability of encountering the substrate. Otherwise, the synthesis of even low levels of the enzyme would be a waste of metabolic energy.

A variation on the limited induction mechanism involves organisms for which the synthesis of the extracellular enzymatic activity is totally inducible, i.e. no extracellular enzyme is synthesized in the absence of its substrate. This condition is advantageous in that no energy would be wasted in synthesis of unnecessary enzyme molecules. A price is extracted in that the response to an influx of metabolizable substrate is slow since *de novo* enzyme synthesis must be induced. Enzyme induction would involve one of the following mechanisms: (i) production of soluble inducer molecules could be the result of activities of coresident microbes in the microsite. This would be an example of commensulism. (ii) Inducers could be

yielded by nonbiological catalysis of the substrate. Some substrates may be decomposed chemically into intermediates that could stimulate enzyme synthesis. (iii) The inducer could be produced by death and lysis of a proportion of the population. In this case either the inducer or enzymes necessary to yield it are released from the cell. (iv) Lastly, the inducer could be produced through action of the humic acid fraction, i.e. catabolism of the enzyme substrate by the stabilized enzymes present in the soil microsite.

Other potential adaptive mechanisms involve physical limitation of the distance the enzyme could migrate from the cell. This adaptation includes enzymes that by definition are not classified as extracellular enzymes, but they certainly are synthesized for external activity. Activities grouped in this category include periplasmic enzymes as well as those linked to cell walls or retained in cell polysaccharide layers. Linkage to the cell of externally active enzymes requires cellular adaptations to optimize the potential for the cell to encounter the substrate. For this mechanism to be successful, the microbe must be in direct contact with the substrate. Generally, this mechanism would be advantageous to the motile organism capable of a chemotactic response to available substrates. This adaptation is commonly observed with plant cell wall decomposers, for example colonies of cellulolytic bacteria growing on the surface of plant debris.

6.7 Concluding Comments

Soil enzymes have been shown to be useful, if not essential, tools in evaluating soil life processes. When properly applied, enzyme assays reveal the nature of the biological processes occurring in a particular soil and the impact of external forces on these transformations. Interpretation of data is limited by our capacity to relate activity measured in the laboratory-manipulated soil sample to conditions at the actual field site; yet, with a proper appreciation for soil heterogeneity and the limitations to biological processes occurring therein, such analyses provide a useful depiction of the soil system. Such data can be useful in evaluation of biogeochemical process rates *in situ*, determining the impact of reclamation management on microbial community recovery, and estimation of soil quality.

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7

Microbial Interactions and Community Development and Resilience

Soil is an harsh habitat for microbial community development. A variety of common soil physical and chemical properties exists that singly or in combination could easily preclude or minimize microbial growth and activity. These physical and chemical conditions of the soil ecosystem may appear to provide a sufficient barrier to invading microbes to prevent their colonization of a soil ecosystem. Interestingly, as long as life-precluding extremes in physical and chemical properties are not encountered by invading microbes, it is the biological community that frequently determines the ultimate destiny of the alien organism. That is, the invader may be able to adapt to the physical and chemical environment, but dealing with the competitive and defensive mechanisms of the indigenous community is more problematic. This conclusion is based on the long-understood fact that microbes do replicate when introduced into sterile soil samples (e.g. Conn and Bright 1919; Katznelson 1940). Although the microbial population may be functioning at the margins of their acceptable physical and chemical limitations in a sterilized soil sample, if all the metabolic and physical requirements of the microorganism are met, growth occurs.

Typical data exemplifying this principle are provided by an evaluation of the behavior of *Escherichia coli* in a sterile organic soil, Pahokee muck (Figure 7.1) (Tate and Terry 1980). With initial populations of approximately 1.0×10^5 propagules g^{-1} dry soil, more than a 100-fold increase in population density resulted. Note that when large populations of bacteria were added to the soil, growth was limited or prevented, as was shown when bacterial populations of greater than 3.0×10^7 propagules g^{-1} dry soil were added to muck samples. In this case, the population density of the added *E. coli* in the sterile soil remained stable over the 10-day incubation period. The limitation to cellular replication when excessive population densities are added to soils samples may result from space limitations (i.e. the physical space for cellular replication is not available) or from biological interactions between the soil-amended bacterial propagules themselves inhibiting

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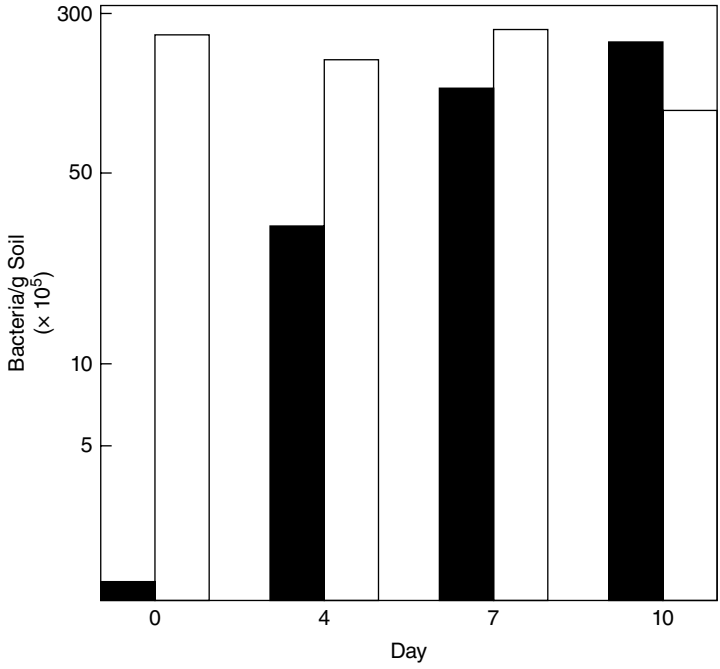

Figure 7.1 Survival and growth of *Escherichia coli* in sterile Pahokee muck. The shaded bars represent soil samples amended with approximately 1.1×10^5 bacterial propagules g^{-1} soil whereas the open bars depict soil samples receiving approximately 3.0×10^7 cells g^{-1} soil.

or preventing growth. Furthermore, it should be noted that over the duration of the study (10 days), a significant decline in the microbial populations did not occur in sterilized soil.

These laboratory observations should not be surprising, since colonization of sterile soil habitats or sites with low population densities must be assumed *a priori* to occur naturally. The most obviously related situation, i.e. colonization of a newly established, essentially sterile soil site, is the invasion and colonization of nascent soils, such as volcanic ashes. Conditions may be extremely stressful, especially in newly formed volcanic soils, but as the physical and chemical limitations to microbial growth are ameliorated (e.g. surface area increased through fragmentation of rocky structure, incorporation of fixed carbon and nitrogen as the result of growth and death of pioneer microbial communities), extensive microbial population densities and the intricate microbial communities characteristic of mature soil ecosystems do develop.

These somewhat simplistic laboratory observations and rare-case field situations do not represent the most common experience involving behavior of invader

populations in native field soils. When mature field soils are amended with foreign microbes (such as through application of sewage effluent), the introduced organisms generally quickly die. Data exemplifying this natural phenomenon are easily recovered from an examination of the public health literature. For example, Reddy et al. (1981) noted half-lives for fecal coliforms amended to a variety of soil samples of 2–150 hours, for *Salmonella* sp. of 2–185 hours, and for poliovirus of 7–416 hours. This short-term survival of microbes foreign to the soil community is fortuitous for purification of pathogens from societal waste materials and has provided the scientific justification for approval of land-based sewage and similar waste material disposal methods.

In contrast, the failure of soil-amended microbial populations to survive for extended periods is more troublesome in situations where development of new microbe-based catabolic activity in a soil site is desirable. This situation can be exemplified by the efforts to develop genetically engineered or laboratory selected bacterial strains for use in soil renovation procedures. The limited probability of survival and/or activity of the laboratory-derived or -selected microbial strains when inoculated into the contaminated soil site raises a significant concern regarding the practicality of soil bioremediation with use of laboratory-produced microbial strains.

Since alien organisms readily survive when added to sterile soil samples, it is logical to conclude that the primary barriers to establishment of exogenous microbial populations in soil relates to a limited capacity for the introduced organisms to cope with preexisting soil populations. This minimal survival of alien propagules in soil may involve a variety of biological exclusionary mechanisms, including the inability of the invader organisms to compete successfully with indigenous populations; sensitivity to biologically produced toxicants existing in soil; and susceptibility to common soil predators or parasites.

These contrasting observations between native and sterile soil samples underscore the importance of biological interactions in the determination of the nature of the soil microbial community composition and function. Soil communities are logically in a constant state of flux. As nutrient supplies are exhausted or reduced by microbial community respiration and growth or alternatively expanded through influxes of plant biomass, the stresses on microbial activity and selective forces on microbial communities are continually changing. Thus, situations are readily envisioned where minorities or newly imported members of the community may become more competitive due to the changing ecosystem properties and therefore become major players in community dynamics. The soil dynamic forces controlling these changing community dynamics are the same delimiters determining successful soil inoculation for bioremediation or purification of sewage effluent. Thus, to understand situations where insufficient die-off of undesirable microbes occurs in soil or the contrasting inability to establish desirable microbial species in a soil site,

a clear delineation of the interactions within the total soil community (i.e. viruses, bacteria, fungi, protozoa, nematodes, and higher animals) is required. Thus this chapter is presented with the objective of analyzing the general traits or types of microbial interactions in soil and determination of the impact of these interactions on community adaptation and resilience following ecological insult. This chapter plus Chapter 8 (The Rhizosphere?Mycorrhizosphere) provide a preview of the concepts of soil, plant, and microbial interactions supporting soil biological remediation processes such as those presented in Chapter 16 (Soil Microbes: Optimizers of System Sustainability and Reparation of Damaged Soils).

7.1 Common Concepts of Microbial Community Interaction

To elucidate the basic principles of the interactive nature of the components of the soil biological community, the nature of the soil ecosystem must be appreciated. Thus, a consideration of the terms delimiting an ecosystem and the life contained therein is necessary. Foremost among these relationships is emergence of a common concept regarding the meaning of the statement "ecology of the organism." The word "ecology" is commonly misused by both the general public and the scientific community. By definition, ecology is the study of the relationship of organisms to their environment. This environment includes all abiotic and biotic factors impacting on the cell. Biotic properties of an ecosystem include not only those traits commonly classified by a society as being "natural" but also must include any consequences of anthropogenic intervention or interaction with the site. Although the effects of human activities on a particular soil ecosystem are by definition natural, or are a normal portion of the driving forces in an ecosystem (since people are commonly a component of the aboveground ecosystem), they are usually distinguished in evaluation of ecosystem function. Perhaps this mental separation of anthropogenic factors from that which is generally considered "natural" results from an impression that they can be eliminated, minimized or at least are to some degree managed.

Anthropogenic interactions do alter the properties of the soil biological community and the nature of the interactions between members of that community when they modify a controlling property of the soil ecosystem in sufficient magnitude to shift the equilibrium of the site. This alteration of system descriptors can be exemplified by examining the conditions that must be met for amendment of a chemical to a soil site to alter the biological equilibrium. Three preconditions for effect may be highlighted.

- *The chemical must be added in concentrations that significantly change the levels preexisting in the site*. For example, nitrogen contained in sewage effluent could

be a major nitrogen source to plant communities in land disposal systems. There are notable situations where the nitrogen contained in sewage effluent would be an insignificant contributor to the total soil mineral nitrogen pools. Drained histosols (i.e. organic soils) provide an example where effluent nitrogen would not significantly change the *in situ* soil nitrogen concentration. The soils of the Everglades agricultural area are composed of approximately 85% organic matter, which is in part biodegradable. Decomposition of this organic soil results in the mineralization of about 1400 kg N ha^{-1} annually (see Tate 1980). Thus, addition of a few kg of mineral nitrogen to these soils through sewage effluent disposal would not make a meaningful change in the soil nitrogen concentrations.

- *The chemical must be added in sufficient quantity to alter the steady-state equilibrium*. For example, there is considerable concern regarding the impact of acid rain on environmental systems. In well-buffered, calcareous soils, the impact of the acid content of the rain water is modulated by the buffering action of the calcium carbonate and soil organic matter.
- *The chemical may be toxic to the soil organisms*. The toxic impact may be of short duration (e.g. as might be observed with a readily mineralized organic toxicant) or longer lived (e.g. a biodegradation-resistant toxicant). Biodegradable toxicants temporarily inhibit microbial processes. Examples of biodegradation-susceptible compounds commonly encountered by soil communities are pesticides and petroleum components contained in accidental spills or used as carriers during pesticide application. In contrast, a nonbiodegradable compound would have a more lasting impact on ecosystem function if toxicant-resistant microbial communities are not developed.

Due to the interactive nature of soil biological communities, soil alterations that might be expected to stimulate only a few species (e.g. those capable of mineralizing a specific pesticide added to the soil) may cause reverberations throughout the entire biological hierarchy, due to the simple change in population density of a single group of organisms. For example, significant increases in easily metabolized carbon will increase microbial competition for the new nutrient and energy source. The augmented bacterial population density resulting from the increased energy and nutrient supply will stimulate the activity of secondary feeders, such as bacterial feeding nematodes and protozoa. If sufficient carbon resources are introduced into the soil system, competition for mineral nitrogen or space could be accentuated. Thus, the anthropogenic activity associated with the chemical spill or even the purposeful amendment of the soil ecosystem with biologically decomposable organic matter may alter the biological interactions at all trophic levels.

The impact of an environmental insult on the basic properties of an undisturbed site are commonly of interest at the ecosystem level, but full explanation of the processes occurring therein requires specific targeting of a particular portion

of the soil biota. Interactions may be evaluated at the individual organism, population, community, or total ecosystem levels. For most soil inhabitants, definition of the individual is reasonably simply accomplished – the single bacterial, archael, algal, or protozoan cell. This entity is more difficult to define for filamentous organisms, fungi, and some actinomycetes. For most of these organisms, the total mycelial structure developing from a single spore could be considered to be the basic entity of this ecological hierarchy, the individual.

Above the individual level is the more easily evaluated population. A population is defined as being constituted of all the individuals of the same species or function. More rigorous definitions require a species definition, but in soil microbiology it is not unusual to discuss populations of nitrifiers, denitrifiers, or diazotrophs as well as populations of *Bacillus* or *Pseudomonas* spp. The summation of the populations in a particular ecosystem constitutes the community. As indicated above, the ecosystem is the combination of both the living and nonliving components of the environment.

7.2 Classes of Biological Interactions

In simple situations, for example an axenic culture growing in a laboratory, the microbial population could be said to be controlled by its nutrient supply, moisture (for colonies growing on solid media), energy source, and a variety of other reasonably defined chemical and physical traits defining the ecosystem. But, even in a defined growth medium, the determinants of cultural development extend beyond the more easily described physical and chemical conditions to the more ill-understood biological interactions. The microbes are not alone in their ecosystem. Each individual must deal with the physical presence and activity of their progeny. Even if a microbe enters a new habitat as a single spore and no other organisms are present – a highly unlikely situation – the primary product of the pioneer is its progeny. That is, for successful colonization of the site to occur, the growing, respiring microbe must replicate. At the least, the presence of this new cell results in competition for nutrients and space as well as an increase in waste materials accumulated in the vicinity of the developing microcolony. These parent–progeny interactions are simple in that the phenotypic and genotypic differences between the cells are minimal. Except for any mutations that may have resulted during the division cycle, each cell is essentially identical. Thus, each microbial cell may be considered to have equal capability and opportunity in the competition for available resources.

As the complexity of the community increases, this simplicity is replaced by a variety of more complex, positive and negative interactions. There are few, if any, monocultures in terrestrial ecosystems. Microbes must cope with actions of a variety of

Table 7.1 Biological interactions occurring in soil ecosystems

Type of interaction	Species A	Species B
Positive interactions		
Neutralism	0	0
Commensulism	+	0
Mutualisms: protocooperation	+	+
Mutualisms: symbiosis	+	+
Negative interactions		
Competition	−	−
Amensalism	0	−
Parasitism and predation	+	−

individuals with greater or lesser capability to deal with cohabitants of their microsite. A summary of the classical designation of the types of interaction affecting soil microbial community development is presented in Table 7.1. Each of these processes will be analyzed individually from the view of their importance to soil microbial community as well as to total ecosystem development.

7.2.1 Neutralism

Neutralism is included on the list of interactions of soil microbes for completeness, but it is unlikely that such minimal impacts of an organism on its neighbors occur in a soil ecosystem. By definition, in a neutralistic association the organisms living in the same habitat would have absolutely no effect on their neighbors. The rather simple situation of pioneer communities described above belies the possibility of occurrence of neutralistic interactions of microbes existing in the same habitat. Due to limitations to growth afforded by soil physical structure (space limitation) as well as restrictions resulting from supplies of growth factors, carbon resources, and the generally harsh conditions of soil, such a degree of noninteraction of adjacent populations is highly unlikely.

7.2.2 Positive Biological Interactions

7.2.2.1 Commensalism

In this opportunistic relationship, one organism is able to grow or function as the result of the action of a second organism. The second organism gains no benefit from the relationship. Commensalism is most likely the most common soil microbial

interaction. Examples include such critical soil processes as existence and function of anaerobes, organic matter decomposition, cross-feeding of nutrients, provision of growth factors, and toxin inactivation.

Obligatorily anaerobic bacteria cannot function in the presence of free oxygen. These organisms are highly sensitive to even low levels of molecular oxygen and die quickly in its presence. Clearly, adaptations are necessary for these organisms to exist in an aerobic world. The co-occurrence of aerobes in the mosaic of aerobic and anaerobic microsites of the surface of most soil ecosystems facilitates the development of colonies of strict anaerobes. This enhancement of anaerobic processes is demonstrated by evaluation of microcolony development in and about a soil aggregate. Soil aggregates commonly occur in soil in situations where aerobes are growing on the aggregate's surface and anaerobes are replicating internally. Facultative organisms may exist and function throughout the soil aggregate. For a benefit to be accrued by the internal, oxygen-sensitive anaerobes, populations of aerobes on the aggregate surface must utilize molecular oxygen at rates faster than it can diffuse into the interior of the aggregate. Thus, the anaerobes functioning within the soil aggregate do so at the behest of the rapidly respiring surface-residing populations. Those organisms on the aggregate surface do not necessarily gain any benefit from the internal populations. (The qualification "necessarily" does not pertain specifically to the matter under discussion directly. Conceivable benefits could be derived from the potential for products of the anaerobic metabolism to diffuse to the aggregate surface where they could contribute to the metabolism of the aerobes. Such benefits would only be incidental to the commensual interaction involving molecular oxygen.)

Similar beneficial, gratuitous associations can be noted with the decomposition of plant tissue in soil. Many soil microbes are capable of decomposing the easily metabolized substituents of cellular cytoplasm (e.g. amino acids, proteins, amylose, simple sugars), but those materials are separated from the general soil microbial community by the cellulosic cell wall. Cellulose-degrading microbes disrupt the cell wall, thereby rupturing the cell and making the internal contents available to noncellulytic microbial populations. Since, in general, cellulytic microbes are poor competitors for easily metabolized substrates, the cellulose degraders gain no or minimal benefit or detriment from the activity of those populations stimulated by the release of internal cellular substituents.

Similar metabolically based relationships are associated with production of metabolic by-products and growth factors. A variety of products are yielded by the respiring microbes. These substances may include ammonium as well as a variety of partially oxidized carbonaceous substances, such as a variety of organic acids. For example, methanogens use the organic acids produced from catabolism of such complex organic substances as cellulose by clostridia to produce methane, a greenhouse gas. Additionally, the organic acids may be used by other soil organisms as carbon and energy sources.

Table 7.2 Vitamin-producing and vitamin-requiring populations in soil

Vitamin	Require[a] (percent of isolates)	Producers[b] (percent of isolates)
Thiamine	19.4	35.5–56.7
Biotin	16.4	15.2–32.7
Vitamin B_{12}	7.2	29.9–34.6
Pantothenic acid	4.6	–
Folic acid	3.0	–
Nicotinic acid	2.0	–
Riboflavin	0.6	35.2–67.3

[a] From Lochhead and Burton (1957).
[b] From Lochhead (1957).

Bacteria requiring vitamins (prototrophs) are commonly isolated from soil. In a study of 499 soil bacterial isolates, Lochhead and Burton (1957) found that 27.1% of the organisms required one or more vitamins for growth whereas 63% of these more fastidious organisms required more than one vitamin. The most common essential vitamins required by soil bacteria were thiamine and biotin (Table 7.2). Necessarily, any organism that can grow without an external vitamin source must be synthesizing that vitamin (auxotrophs). Thus, the latter organisms provide the essential substances to the prototrophic population. Most organisms synthesize only the quantities of vitamins required to meet their own metabolic needs. These vitamins become available to prototrophic members of the community upon death and lysis of the auxotrophic cell. Perhaps of more significance to the active soil community is the fact that many auxotrophs excrete vitamins. More than 50% of the isolates studied by Lochhead (1957) excreted one or more vitamins. These latter organisms encourage development of commensual relationships with other soil inhabitants.

A variety of toxic substances that enter an ecosystem through anthropogenic intervention or that are produced internally as by-products of microbial metabolism may have a generalized inhibitory effect on microbial respiration. For example, acidification of a soil site by acid mine drainage or the internal production of sulfate from elemental sulfur or sulfide oxidation (see Chapter 15) results in the inhibition of acid-sensitive organisms. Reduction of sulfate by sulfate-reducing bacteria causes a generalized improvement of ecosystem conditions for the members of the soil community.

7.2.2.2 Mutualism

Mutualism involves an association in which both organisms benefit. Special cases or related terms include protocooperation and symbiosis. In protocooperation, any microbe that can catalyze the requisite reaction(s) can function in the

combination. Thus, the association is neither obligatory nor specific. In contrast, symbiotic associations are obligatory for the function to occur and only specific microbes are involved. A generalized example of protocooperation is a process that could be postulated to occur in any environment. For this example, a soil system is proposed within which neither growth factors A or B are present. Microbes requiring either of these growth factors would not be expected to grow in the system. Yet, both groups of organisms can be found to be active in the absence of their prerequisites for replication. For this combination of organisms to exist in the absence of the requisite nutrients for their grown, the microbial population that requires substance A must produce compound B. Similarly, the substance B-requiring strain must produce substance A. Neither of these organisms would be capable of growth in the absence of the coexisting population, but both can function in combination. The combination of organisms existing in this bimember community is determined by metabolic capacity, not species designation. That is, a third species with the requisite traits could just as easily participate in the development of the community. Specifically, such a system could be envisioned to occur in soils lacking both fixed nitrogen and carbon (newly formed volcanic soils). Successful biological associates in the pioneer community could include a green alga (a photoautotroph) and a nitrogen-fixing bacterial species (a diazotroph).

7.2.2.3 Symbiosis

To some, symbiosis is the most restrictive form of mutualism, whereas for others, it is considered to constitute a separate class of biological associations. Symbiosis is an obligatory, nontransitory, mutually beneficial interaction. This association is essential for either the existence of the organism in the ecosystem or for the occurrence of a particular processes, such as nitrogen fixation. The nature of this association does not mean that either of the partners cannot occur singly, under different circumstances. For independent existence, all the requirements for growth must be supplied. For example, *Rhizobium* sp. and legumes combine in a symbiotic relationship to fix nitrogen. In soil, the association is obligatory for nitrogen fixation to occur, but both the legumes and rhizobial strains are capable of growth separately as long as all of their nutrient requirements are met. In soil, association of the bacteria with legumes is obligatory for nitrogen fixation to occur. In laboratory culture, the rhizobial strain can be induced to fix nitrogen exclusive of the symbiotic association, but this independence is not expressed outside laboratory cultures.

Commonly encountered symbiotic associations in soil systems are the *Rhizobium* sp.–legume interaction, an actinomycete–higher plant nitrogen fixing interaction, and mycorrhizal associations involving plant roots and a variety of fungi.

7.2.3 Negative Biological Interactions

Negative interactions or antagonisms are of interest from a variety of viewpoints. These detrimental associations may explain the failure to successfully inoculate soil communities with propagules of beneficial organisms (including genetically engineered microbes). Also, the principles of antagonistic associations underlie the use of soils as purifying agents for pathogen-containing waste materials and may be exploited in development of biological control methods for control of plant pathogens.

7.2.3.1 Competition

Competition, rivalry over a limiting factor, is generally described in relation to a less active species being suppressed by a second, more vigorous species. In theory, it is considered that one species derives full benefit from the available resources, whereas development of the second population (the weaker competitor) is reduced. Should the competition be sufficiently intense, the poorer competitor is eliminated (*competitive displacement*). Generally, with competition for limiting nutrients, if the supply is continuous, the poorer competitor is eventually displaced.

This "all or none" effect of the competition is clearly not representative of the true field situation. If the weaker population replicates at all, it will exhaust at least a small portion of the object of competition (e.g. carbon, nitrogen, molecular oxygen, phosphorus, space). Thus, the ultimate density of the more successful population as well as its growth rate must be reduced. Furthermore, in natural ecosystems, competition based on availability of a single resource is unusual. A variety of ecosystem properties tend to mitigate the intensity of competitive interactions, thereby allowing coexistence of both competitors (Fredrickson and Stephanopoulos 1981).

An obvious question regarding competition is: What makes a microorganism a good competitor? An understanding of these factors would be useful in such environmentally important projects as developing genetically engineered organisms for bioremediation. An organism with exceptional ability to mitigate environmental hazards is of limited or no value if it is not capable of competing with indigenous soil microbes. Thus, selection of desirable genetic traits in bioremediation inoculants should include survival as well as the desired metabolic capacities.

Several reasons why certain cell properties provide a competitive advantage have been proposed, but none fully explains the outcome of population interactions and community development in all situations. Most likely, there is no single phenotypic microbial trait essential for successful domination of an habitat. Rather, a variety of capabilities contribute and are expressed depending upon the properties of the competitor, the habitat in which they are growing, and the population density-controlling condition. Four properties that clearly are beneficial for

successful competition are (i) growth rate, (ii) efficiency of nutrient utilization, (iii) capacity to catabolize a variety of metabolic substrates, and (iv) motility.

Growth rate can be proposed to be effective in competition for nutrients and space. Succinctly stated, the organism with the shortest generation time, all else being equal, would be the most effective competitor. This property is actually linked to a second mechanism (efficient nutrient utilization) in that efficient energy management is a necessary contributor to an augmented growth rate. The lesser the quantity of energy that must be expended for growth, the greater the potential for a faster growth rate. An excellent means of preserving energy for the microorganism is to avoid the unnecessary synthesis of enzymes. For example, an organism that synthesizes its own growth factors even in the presence of adequate levels of those substances would necessarily have its growth yields reduced by the quantity of energy used in synthesizing the growth factor and the requisite enzymes.

Substrate versatility and motility both offer contrasting advantages to the competing microbes. Less fastidious members of a community escape competitive limitations by catabolizing alternate carbon and energy resources. This capacity to utilize a second carbon or energy source once the original substrate has been exhausted would provide a competitive advantage for molecular oxygen and space competition. Motile organisms reduce their competitive pressures by migrating to another microsite. Environmental factors can determine the outcome of a competitive interactions (for example, see Rosenzweig and Stotzky 1979). In a competition between the fungus *Aspergillus niger* and the bacterium *Serratia marcescens*, amendment of soil with 3% kaolinite reduced the antagonism and higher concentrations of the clay totally eliminated it. The antagonism was also influenced by soil pH and carbon availability (Rosenzweig and Stotzky 1980). The data suggested a direct correlation between the degree of inhibition and the rate of glucose utilization by the bacteria, indicating that the antagonism resulted from competition for carbon. This competition was influenced by the clay content (kaolinite) of the soil and the soil pH.

7.2.3.2 Amensalism

Amensalism is the suppression of the growth of one organism by products of growth of a second. This growth inhibition may result from as simple a situation as alteration of the soil pH or it may be derived from production of a growth-inhibiting or lethal biological product (e.g. antibiotics). For example, *Thiobacillus* spp. commonly reduce soil pH through the oxidation of sulfide to sulfate. Since the pH may reach values as low as 2, the growth of any pH-sensitive microbes is inhibited.

Two major types of biological inhibitors or toxins are produced by soil microbes: those effective at high concentrations (organic acids, chelators) and those inhibitory at low concentrations (antibiotics). The growth-controlling impact of the former compounds in soil has been reasonably well accepted, since the substances

can be easily quantified in soil samples and their interactions with soil microbial populations easily shown. The role of antibiotics within the soil ecosystem is more problematic.

A conceptual barrier exists that has, for some time, prevented appreciation of the impact of antibiotics in complex, heterogeneous systems such as soil. When a soil system is examined as a whole, it is difficult to demonstrate either the presence or activity of any antibiotic *in situ* at concentrations inhibitory to microbial growth. Although the antibiotic may be present in inhibitory concentrations in a soil microsite, averaging this localized concentration with that portion of the soil sample containing little or no antibiotic leads to an underestimation of the potential impact of these microbial products *in situ*.

Further conceptual difficulties are encountered when it is noted that in culture, antibiotics are commonly produced by microbes in a resting stage following a period of rapid growth. Since one could reasonably hypothesize that the primary benefit of antibiotics would be derived during active growth, it is difficult to conceive of a role of these substances in native soil sites. This hypothesis is developed from the consideration that during growth, the microbes would be involved in intensive competition for space and nutrients. There are other opportunities for microbes to benefit from antibiotic production. Resting cells could gain protection from predators. Also, during outgrowth of spores, the antibiotic could suppress susceptible members of the community sufficiently to allow the nascent microbes to become established. That is, the antibiotic could provide a release from competitive pressures. This possibility has been shown for streptomycin production by *Streptomyces griseus* (Szabo et al. 1985). Streptomycin was synthesized by vegetative mycelia and bound by spores. Release of the antibiotic from the spores during germination was proposed as a competitive advantage for the young hyphae in the microenvironment.

A simple argument in favor of the role of these substances in soil relates to the fact that a major portion of the soil community is capable of producing antibiotics. The complexity of the molecules suggests *a priori* that the substance would not be synthesized without some selective advantage being conferred on the producer. The logic of this hypothesis is clear, but its truth in complex soil systems is difficult to prove or disprove.

At this point, it must suffice to say that gains from antibiotic production may be accrued at the point of cell-to-cell contact. Furthermore, although no obvious macrosite effects can be documented, the effects of community function observed at the macrosite level are clearly the product of the intensity of competition at the microcolony level. That is, although direct effects of antibiotics at the total system level are difficult to demonstrate or quantify, any impacts of the antibiotics at the microsite level would have at least some effect on the system as a whole.

The actual importance of antibiotics in native ecosystems is still conjectural but their potential has been clearly shown in laboratory, greenhouse, and field studies.

For example, *Streptomyces olivocinereus*, which produces the antibiotic heliomycin, was shown to be antagonistic to *Arthrobacter crystallopoites* in laboratory media and in sterile soil (Polyanskaya et al. 1983). Also several antibiotic-producing bacterial strains tentatively identified as *Bacillus subtilis* and a fungus, *Penicillium nigricans*, protected onions from infection with *Sclerotium cepivorum* in native muck soil in a controlled environmental chamber. These organisms also reduced the occurrence of the resulting disease, onion white rot, when used as a seed treatment in the field (Utkhede and Rahe 1980). Similarly, amendment of the antibiotic streptomycin or streptomycin producers to the *Rhizobium* inoculum for soybeans (*Glycine max* L. Merrill) and alfalfa (Medicago sativa L.) increased nodulation and plant biomass (Hossain and Alexander 1984; Li and Alexander 1986, 1988). Thomashow et al. (1990) found that inoculation of wheat roots with antibiotic-producing (phenazine-1-carboxylic acid) *Pseudomonas fluorescens* resulted in antibiotic synthesis in the rhizosphere of the wheat plants and a reduction in the occurrence of take-all disease caused by *Gaeumannomyces graminis*.

Another group of biologically synthesized compounds that appear to be useful in reducing plant disease through antagonism of pathogens are siderophores (see Leong 1986 for a review of this topic). These substances appear to be active at higher concentrations than is characteristic of antibiotics, but when they result in suppression of microbial growth at low concentrations, they can be classed as antibiotics. Siderophores are extracellular, low molecular weight (500–1000 dalton) iron-transporting compounds synthesized by a variety of microorganisms growing under low iron conditions. These substances selectively complex ferric ion with a high affinity, thereby reducing iron availability to competing organisms. Most commonly studied siderophore-synthesizing microbes from the view of controlling plant pathogens are members of the *P. fluorescens-Pseudomonas putida* group. Inoculation with a variety of *Pseudomonas* strains appears to be useful in controlling *G. graminis* var. *tritici* (Hamdan et al. 1991; Thomashow and Weller 1990) and *Rhizoctonia solani* (de Freitas and Germida 1991) on wheat. Siderophores may also be instrumental in reduction of pathology in disease-suppressive soils (see Kloepper et al. 1980 as an example of these studies and Hornby 1983 as a review of suppressive soils).

7.2.3.3 Parasitism and Predation

Predators and parasites, organisms that feed upon living biomass, also play a key role in the soil ecosystem. Soil bacterial and fungal populations are capable of producing resting structures or of entering resting stages (metabolic states where respiratory and metabolic activity are reduced to the minimal level necessary to maintain cellular integrity). Thus, without a means of encouraging the recycling of these nutrients contained in the resting cell biomass through the biogeochemical cycles, essential nutrients (e.g. carbon, nitrogen, phosphorus) as well as space

could be retained in or by these structures to the detriment of more active soil organisms. Facultative predators encourage the cycling of growth substances and liberation of space through parasitic and saprobic consumption of microbial biomass. Parasites and predators maintain the soil bacterial and fungal populations in an active state and enhance nutrient cycling between soil reservoirs through consumption of microbial biomass. This feeding activity of predators and infectivity of parasites maintains a younger, more active, soil microbial population.

Essentially, all types of predators or parasites are present in the soil ecosystems. Bdellovibrios, bacteria which prey on other bacteria (e.g. Casida 1980, 1983); bacteriophages; protozoa and nematodes are all active in soil ecosystems. These organisms may ingest their nutrients by consuming intact cells (*holozoic feeding*), as is commonly described for protozoa, or by producing extracellular enzymes, which lyse other bacteria, fungi or algae. This latter process is exemplified by the predatory bacterial populations.

A key consideration in evaluation of predator or parasite behavior in any ecosystem relates to the observation that both the host and parasites or prey and predators coexist in the same ecosystem. Thus, it may be asked, "Why do predators and parasites not totally eliminate their prey and hosts?" A variety of laboratory studies have been conducted to elucidate the causes for coexistence of prey and predators. These studies generally involve incubation of a suscept with a predator (e.g. Danso and Alexander 1975) or a parasite (e.g. Wiggins and Alexander 1985) in a liquid culture. Most analyses of the specific survival adaptations outlined below were derived from study of these simplified microcosm type interactions. It is commonly noted in these studies that the prey populations achieve an equilibrium density of approximately 10^6 propagules mL^{-1} of culture. (Furthermore, in the Wiggins and Alexander (1985) study, a minimal host population of approximately 10^4 colony-forming units mL^{-1} was required for bacteriophage replication.) If prey population densities significantly greater than 10^6 propagules mL^{-1} are amended to the test system, they decline to about 10^6 propagules mL^{-1}. In studies where low prey densities were incubated in growth-supporting media in the presence of a predator protozoan population, the prey population increased to the limiting population density of about 10^6 propagules mL^{-1} culture which was subsequently maintained.

Clearly, some mechanism must preclude total elimination of susceptible populations. Otherwise, eventually both groups of organisms would cease to exist. Extinction of prey would result in starvation of the parasite or predator. Some exceptions to this observation are noted. The continued survival of a "food" reservoir is essential for obligate predators, but facultative organisms are freed of this limitation in that they can use living prey or consume organic matter saprobically. Some relief from this limitation to parasitic or predatory behavior is also gained by the organism that can consume a variety of host or prey species. Once one, perhaps even the

prime, food source becomes limiting, consumption of an alternate food source not only spares the original prey population but also reduces the energy expended by the predator in search of food, since it is reasonable to assume that selection of the host is driven to a significant degree by the probability of encounter.

A variety of mechanisms have been proposed to explain the balanced attack by parasites or predators (for a detailed review of this subject, see Alexander 1981). Examples include the following.

- *Interactions among predators*: Increased predator density due to localized consumption of the prey may result in competition among the protozoa. This interference with grazing due to frequent contact among the animals could actually result in cannibalism of the predator population. This means of population control may occur in native sites, but it is not the primary mechanism in that in laboratory studies, amendment of stabilized bacterial–protozoan systems with more bacteria causes further increases in the protozoan population densities.
- *Predators under biological control*: With biological control of predator/prey or parasite/host associations, the predator and parasite populations may be controlled by populations that feed on them directly or by biologically formed toxins. Because of the complexity of the biological community in soil, it is likely that organisms that feed on bacterial predators or parasites would commonly occur. Again, since stabilization of predator–prey populations occurs readily in model systems with one protozoan and bacterial species present, feeding on the bacteriovorous organisms by higher organisms cannot be the sole explanation of population stabilization.

  Toxic interaction is an attractive hypothesis, but insufficient data have been accumulated to allow its evaluation.
- *Genetic feedback*: The primary assumption behind this mechanism is that a spontaneous mutation and population selection can occur. These mutations would result in changes in the prey cells that would reduced their susceptibility (desirability *per se*) to the predator. This mechanism is certainly a plausible adaptation of the prey population yet, at least in short-term laboratory cultures, such selection appears not to occur. In studies of the interactions of *Rhizobium meliloti* and the protozoan *Naegleria*, the surviving population appeared to be as susceptible to protozoan attack as the original population encountering the predator (Danso and Alexander 1975). In contrast, development of bacteriophage-resistant cultures through modification of receptors on the bacterial cells is commonly observed. Thus, it is logical to assume that modification of the bacterial cell wall or even production of chemoattractants could reduce interactions with predator populations, but this has yet to be demonstrated in laboratory studies of predator–prey interactions.

- *Refuge*: Neither soil or aquatic ecosystems are homogeneous. Both systems are composed of mixtures of particulates (detritus in aquatic systems) intermixed with a somewhat contiguous aquatic system or water layer. Thus, predators could be physically screened from the presence of prey. Pores between the particulate matter can easily be envisioned to be of a size through which the predator cell could not pass. Thus, even though its food source may be within a few microns of its cell, the predator may die. The more heterogeneous the environment, the greater the probability of the occurrence of refuges. This is particularly true of soils since a vast diversity of pores of varying sizes interconnect the various microsites suitable for microbial growth. Unfortunately, since survival of bacteria occurs in both soils and liquid cultures, refuges are not the universal mechanism for prey survival in soil, but it is certainly logical to propose some importance for this mechanism. Evidence that refuges can occur was provided in a study of a model system where *Paramecium caudatum* was protected from *Didnium nastum* by refuge.
- *Switching*: Most predators are capable of consuming a variety of prey species, although one or a few may appear to be more desirable. For example, Casida (1989) noted that nonsoil bacteria (*E. coli* and *Bacillus mycoides*) amendment to soil samples resulted in protozoan population density increases. In contrast, no response in the predator population was detected following amendment of soil samples with the native bacterial species *Arthrobacter globiformis*. Should the predator exist in a complex community, the random attack of a variety of bacterial species could result in less stress of each individual species or strain. No volition of the predator is implied in this process. The statistical probability of random encounter with the various species present in the system results in a reduction in destruction of any single population.

  An exception to this probabilistic evaluation of the occurrence of this switching prey concept was suggested by Mallory et al. (1983). They observed that if alternative prey population densities were above the threshold for active predation and the growth rate of the prey was less than the predation rate, then elimination of the prey species could occur in their microcosm study.

  Again, the data to support switching as a mechanism for survival of prey cells are scant. Logic suggests that in a soil ecosystem, there may at least be a contribution of this probabilistic sparing of microbial cells from attack. Contributing factors in soil are low microbial growth and predation rates as well as limited population densities of all species.
- *Density dependence*: As with the above sparing mechanism, density dependence relies on the probability of the encounter of the predator and the prey. The higher the density of each population, the greater the probability of feeding. Danso and Alexander (1975) noted that at high population densities of *R. meliloti* cells in a defined cultures, final densities of the predators (*Hartmanella* sp., *Naegleria* sp.,

and *Vahlkampfia* sp.) were proportional to the initial prey populations. The rate of elimination of the prey cells was proportional to initial abundance of the predators, but the final populations of both predators and prey were independent of this number. This observation leads to the conclusion that although many parameters of the feeding curve are determined by participant population densities, the population density of surviving bacteria is not. Similarly, in a study of *Bdellovibrio* feeding on a bioluminescent bacterial prey, Varon and Zeigler (1978) found that a minimal population of $3.0 \times x10^6$ prey mL^{-1} culture was required for *Bdellovibrio* cells to have at least a 50% chance of survival. A prey density for development of population equilibrium in their system was 7×10^5 cells mL^{-1} culture. It appears that once the prey density has been reduced to the point where energy expenditure in food acquisition exceeds that returned by prey consumption, sparing of the prey population occurs. That is, the prey population density must be sufficient to provide the requisite energy for the predator or parasite to replicate and search for new food supplies. A population of 10^6 cells mL^{-1} of liquid culture apparently approaches that minimal density for adequate energy return to the feeding organisms. Density-dependent sparing of prey cells likely contributes at least a portion of the survival of suscepts in native environments.

- *Replication to compensate for killing*: With high population densities of host cells, the probability of encounter between them and predators or parasites is reasonably high. Thus, a reasonably rapid population decline is anticipated to occur. But as the host populations recedes, the rate of destruction is reduced simply because of the lower probability of encounter of feeders and host or prey cells. Should the latter populations be replicating, a point is reached where the rate of predation or parasitism is equivalent to the rate of growth of the prey. Considering the low bacterial growth rates commonly occurring in soil, replication to compensate for killing would only be significant in situations with low predator or parasite stress, such as would be associated with low feeder populations.

It is clear from analysis of these hypothesized means of controlling suscept population densities in soil that no single explanation is adequate to answer our initial question of "Why do predators and parasites not totally destroy their prey and hosts?" All of the mechanisms proposed are logical. Although specific ecosystems can be sited where they are not functional, environmental situations can be proposed wherein they would be important. Data invalidating an all-inclusive role of a particular survival adaptation in sparing of suscept populations have generally been derived from study of two or three member liquid cultures. These microcosms, *per se*, are special cases. In themselves, they lack the complexity of a soil microsite. Thus, a particular survival adaptation dismissed in the liquid culture may be crucial for continued existence of individual, predator-stressed microbial

populations. Thus, the final conclusion regarding host or prey survival in soil is that a variety of properties of both the feeding populations and their food source contribute to the equilibrium population densities reached. The exact mechanisms contributing to this process vary with species involved and the nature and properties of the individual microsites wherein the interactions are occurring.

7.3 Trophic Interactions and Nutrient Cycling

To this point, this analysis of microbial interactions has been concentrated at the somewhat simplistic level of single or bilevel trophic interactions, that is, bacterial population – bacterial population or bacterial populations – protozoan community interactions. Clearly, all trophic levels of the soil biological community contribute to ecosystem development. This loosely associated cooperation is best illustrated by examining the trophic interactions involved with oxidation of plant biomass and some of the factors limiting the process rates and population densities. (For a more comprehensive evaluation of trophic level interactions in soil, see Anderson 1988; Anderson et al. 1985 [invertebrates]; Bamforth 1988 [protozoa]; Freckman 1988 [nematodes]; Visser 1985 [invertebrates]; Usher 1989 [arthropods].)

7.3.1 Soil Flora and Fauna

Although microbiologists tend to concentrate their attention on the biochemical contributions of bacterial and fungal species (and to some degree actinomycete populations) to organic matter decomposition in soil, the overall rate of this process is dependent upon viability of the protozoan, nematode, microarthropod, and animal populations indigenous to soil. Although considerations of specific biochemical transformations and their rates are generally emphasized in evaluation of organic matter decomposition in soil, without the contributions of higher organisms (many of which are only indirectly related to organic matter transformation), nutrient cycling in most ecosystems would be severely limited. This is best exemplified by listing and evaluating some specific roles for protozoa, nematodes, mites, and other animals in soil ecosystems.

For decomposition of plant debris to occur at rates essential for the return of mineral nutrients contained therein to the aboveground biomass, it must be populated with soil microbes. This occurs through mixing of the debris within the soil profile as well as by inoculation of the plant litter retained on the soil surface. Mixing of organic matter within soil profile is accelerated by the activity of earthworms, ants, termites, and even burrowing animals. For example, by collecting surface litter and storing it within their nests, termites and ants may create islands of plant debris within a sea of nearly litter-free soil. The biodecomposition of this

organic matter alters plant nutrient availability, soil structure, and soil humic and fulvic acid composition (e.g. see Anderson and Wood 1984; Gupta et al. 1981; Pomeroy 1983; Wood 1988; Wood et al. 1983). This localized improvement of soil properties is revealed by studies of tropical savanna soils (Lee 1974; Ofer et al. 1982). Ofer et al. (1982) found that harvester ants in semi-arid pastures enhanced plant growth in the vicinity of the mound, increased soil humic and fulvic acids, and altered the distribution of organic nitrogen within the various colloidal soil organic matter fractions. For a more detailed evaluation of the role of ant and termite activities in tropical soils, see Anderson and Flanagan (1989).

A contrasting situation is found in ecosystems where the plant litter remains distributed in a surface organic layer, as is commonly observed with temperate forest soils. Since the primary decomposers of plant debris are soil microbes, stimulation of the decomposition rate results from inoculation of the litter by animal movement between the A horizon soil and the litter layer. This transfer of microbial propagules may be accomplished by a variety of organisms including earthworms, mites, and ants. Generally, the inoculation of plant debris results from passive transport of the microbial propagules on the surface of the animal body.

Soil animals may also modify the soil structure in a manner that stimulates organic matter decomposition. The activity of aerobic soil microbes may be controlled in part by the rate of oxygen diffusion into soil pores or by saturation of the pores with moisture due to low water infiltration rates. Each of these limitations may be reduced by earthworm tunneling and ant hill construction. Soil structural modification by earthworms and the resulting impact on plant residue decomposition are exemplified by studies of Zachmann and Linden (1989). Treatment of surface-applied corn residues with earthworms (*Lumbricus rubellus* Hoffmeister) resulted in more rapid residue decomposition and altered water balance of the soils.

As was indicated in Chapter 5, surface area can be a primary controller of biological decomposition. This is particularly acute with plant biomass. A primary indirect means of stimulating microbial decomposition of leaf tissue and frass is to increase its surface area. Soil animals both directly (through their own feeding) and indirectly (through physically breaking the plant structure by their passage through the litter layer) accelerate break-up of the biomass physical structure. Clearly, even higher animals, including human activity, accelerate breakdown of the plant physical structure.

A variety of soil animals directly catabolize soil organic matter. The contribution of these organisms to total organic matter respiration in soil is generally minuscule compared to the activity of soil bacterial and fungal populations, but it may be significant. Elliott et al. (1988) suggest that protozoa, nematodes, and microarthropods may in concert contribute up to 40% of the nitrogen mineralized in North American grasslands. Populations instrumental in this process include earthworms and isopods. For example, the woodlouse *Porcellio scaber* feeds on

decaying pine needles. Its activity is primarily limited by the structural toughness of the needles themselves (Soma and Saito 1983).

Probably, the major contribution of trophic levels above soil bacteria, fungal, and actinomycete populations to soil organic matter cycling results from their use of the primary feeders (bacteria, fungi, actinomycetes) as food sources. These secondary feeders include protozoa and nematodes. This feeding on the primary feeders has two effects on soil microbial activity.

- *Augmentation of nutrient cycling*: As exemplified by nitrogen mineralization (Woods et al. 1982), nutrients yielded from mineralization of organic nitrogen sources in soil are usually incorporated into the microbial biomass first. These sequestered nutrients are not made available to other participants of the biological community until the microbial biomass is mineralized. This is accomplished by the action of soil predators.
- *Stimulation of microbial activity*: The secondary feeders stimulate microbial growth, thereby keeping the primary organisms in a more active state (e.g. Anderson et al. 1981). The activity of the secondary feeders can increase the rate of metabolism of soil organic matter by keeping the bacterial population young and active but it must be remembered that the total amount of carbon mineralized does not change. All that is altered is the time frame over which the organic matter is decomposed. The quantity mineralized is controlled more by its degradation susceptibility and the quantities present.

Alteration of the activity of secondary feeders can also result in a reduction in the rate of organic matter decomposition. In this situation, the primary population is reduced sufficiently that mineralization is reduced. Santos et al. (1981) and Santos and Whitford (1981) examined the metabolism of creosote litter in desert soils by a community composed of microarthropods (tydeid mites), bacteriophagic nematodes, and bacteria. Destruction of the mite population resulted in an increase in their food source, the nematodes. This increased nematode density created greater stress on the bacterial population upon which they were feeding. The resultant decline in bacterial density was expressed as a reduction in litter decomposition. In contrast, elimination of both secondary feeders, nematodes and mites, increased bacterial numbers and litter decomposition. Therefore, it could be said that the mites were effective in controlling litter decomposition by indirectly stressing the soil bacterial population. Lower mite populations lead to reductions in the nematode density which in turn increased bacterial population densities and organic matter mineralization. Similarly, Grant et al. (1983) found that destruction of the invertebrate ostracod populations in wetland rice fields resulted in a 10-fold increase in nitrogen fixation rates, which results from the parallel threefold increase in blue-green algal populations upon which the ostracods were feeding. Previous work by Grant and Alexander (1981) indicated that the selective

feeding traits of the ostracod could be exploited to increase nitrogen fixation in flood soils. They found that animal size and temperature affected feeding rates of the animals, as did cell age of the cyanobacteria. (See Ingham et al. 1985 for further analysis of bacterial, fungal, and nematode interactions in the soil community.)

7.3.2 Earthworms: Mediators of Multilevel Mutualism

To conclude that the summation of the effects of biological interactions in microbial communities is strictly limited to population control and invigoration plus nutrient supply interactions would be to totally miss the beauty of the intricacy of interactions of the macrobiological world. Not only do higher plants and animals provide nutrients to microbes, but they engineer the soil structure in a manner that enhances life function in both realms and optimizes the mutualistic interactions at all trophic levels. An appreciation of the interdependent nature of plant roots and microbes in the rhizosphere is gained through study of Chapter 8. This analysis is concentrated on elucidation of the impact of earthworms as ecosystem engineers and as major controllers of total community function.

An initial insight into the complexity of earthworm biology in soil is gained when it is noted that earthworm species are grouped into three major categories based on their site of activity. Endogeic species are active in mineral soil layers, epigeic species in surface litter, and anecic species move between deeper soil layers and soil surface litter (Coleman and Crossley 1996). Thus, the direct impact of each earthworm species on ecosystem function depends on its site of residence and activity. Endogeic species are major soil or ecosystem engineers because of their production of casts, galleries, burrows, and chambers (Fragoso et al. 1997). A less obvious effect of earthworm activity on soil structure is the potential to increase the quantity of macroaggregates in soil. For example, addition of low densities of *Pontoscolex corethruus* in Peruvian Amazonian soils caused an increase in macroaggregates and a decrease in the proportion of small aggregates (Alegre et al. 1996; Blanchart et al. 1997; Gilotvillenave et al. 1996; Pashanasi et al. 1996). The region of the soil where soil is penetrated by earthworm burrow walls is commonly referred to as the drilosphere.

Soil community benefits easily ascribable to modification of soil structural elements – both aggregation and increased macropores – by earthworms in the drilosphere include increased water infiltration and gas exchange between the soil and atmosphere. Enhancement of aerobic microbial activity by the greater potential for import of molecular oxygen and export of carbon dioxide is clear. Also, the gains in water movement could both reduce the incidence of water-saturated conditions (resulting in waterlogged soils) and increase the amount of water stored in the soil during drying conditions. Less obvious are the effects of earthworm behavior on

organic matter mineralization and community interactions in the drilosphere. Evidence that earthworms increase the rates of plant nutrient generation through enhancement of organic matter mineralization is seen through increased total phosphates in earthworm casts (Bossard et al. 1996), and increased maize production in the presence of endogeic earthworms (Gilotvillenave et al. 1996).

Görres et al. (1997) examined soil directly affected by the anecic earthworm (*Lumbricus terristris*) and found that carbon mineralization was enhanced. Interestingly, microbial biomass carbon was decreased and nematode populations were augmented. These authors suggested that the indirect effect of the earthworms was to stimulate nematode populations and their resultant interactions with the microbial biomass within the drilosphere. The feeding of the nematodes would reduce the bacterial populations, thereby allowing for active growth of the latter. The actively growing bacteria would be expected to mineralize more organic matter than the less active populations, i.e. those not affected by nematode feeding.

7.4 Importance of Microbial Interactions to Overall Biological Community Development

In the above analysis, a dissection of system function has occurred. Truly, these essentials of the soil system cannot exist, and must not be studied, in isolation or even as isolated pieces of a puzzle, *per se*. They must be assembled into a conceptualization of the working whole. Indeed, the ultimate importance of any biotic or abiotic trait or interaction is its contribution to community stability and resilience; that is, the capacity of the ecosystem to be maintained and its resistance to disruptive perturbations. Classically, this stability of the soil community is defined as *homeostasis* (the tendency of a biological system to resist change and to remain in a state of equilibrium).

Maintenance of the equilibrium that defines the existing ecosystem conditions is most commonly accomplished by *negative feedback* (a change in one or more populations induced by a modification of some environmental variable that brings about a response in other populations in such a manner that the original fluctuation is opposed or damped). Negative feedback could be said to be a biological equivalent to the physics action–reaction principle.

Applicability of the principle of negative feedback to maintenance of community stability can be exemplified by a hypothetical situation wherein a biodegradable toxin is added to a soil community (Figure 7.2). The system is defined to be composed of three biological groups – toxin-sensitive bacteria, protozoa capable of feeding upon the bacterial populations, and bacteria that decompose the toxin. For this example, it is not necessary that the toxin be mineralized, but rather a simple detoxification would be sufficient to allow return to the steady-state condition. Note that

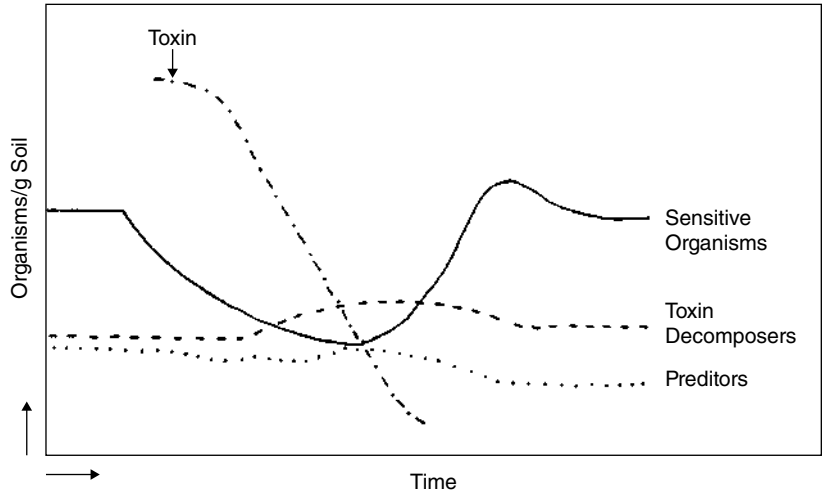

Figure 7.2 Adaptations in a generalized microbial community to reestablish conditions existent prior to input of a biodegradable toxicant.

the initial reaction to the toxic situation is a decline in population densities of those organisms sensitive to the toxin. Because of the time necessary for induction of the enzymes associated with detoxifying the aggravating amendment, a delay in toxin destruction is commonly noted. Increases in the population of toxin degraders can result from utilization of the toxin for carbon or energy. Similar increases in toxin decomposer population densities may result if they derive their energy from the biomass of the killed toxin-sensitive populations. In either situation, a finite quantity of carbon and energy is available for growth of the toxin degrader populations.

Once the toxin has been removed from the system, the toxin-sensitive populations may again reproduce. At this point, conditions favor return to preinsult equilibrium population densities, but a further induction or negative feedback process must occur – protozoan populations increase to "assist" in the return of toxin degrader population density to preamendment levels. With the loss of the nutrient source (exhaustion of the toxin or depletion of the biomass of cells killed by the toxin), the toxin decomposers cease to grow. Protozoan activity facilities the return to steady-state levels by consumption of the excess biomass. With time, the preamendment equilibrium can be reestablished. This is in reality an idealized situation in that it relies upon (i) the toxin being totally destroyed, (ii) sensitive populations not being totally eliminated by the toxic material, and (iii) no permanent change in the controllers of population densities.

The overall manifestation of soil microbial populations may appear to represent a complete return to the preexisting equilibrium, but in reality, it would be more common for the system to move to a new equilibrium condition approximating

the pretoxin insult conditions. A visible manifestation of the interaction at the microlevel on the total ecosystem would not occur unless the newly established microbial community differed from that which preceded it sufficiently to alter overall system properties.

7.5 Management of Soil Microbial Populations

With anthropogenic intrusions into native sites as well as intensive management of developed soil systems, alteration or control of the nature of the soil microbial community is becoming more desirable, if not, obligatory for system survival. Common soil microbial population modification considerations range from alteration of dominant microbial strains (e.g. replacement of indigenous *Rhizobium* sp. strains with varieties possessing more efficient nitrogen fixation capacities) to enhancement of the activity of specific biological decomposition capabilities (e.g. petroleum degraders in oil-polluted soils). In either of these situations, the underlying question regarding success of the inoculation or site management techniques rests at least as much, if not more, on the competitive abilities of the alien organism as on possession of the phenotypic capacity to achieve the desired ends of the project.

Most commonly, generalized soil inoculation has been a failure. Whether it is the use of free-living nitrogen-fixing microbes (e.g. *Azotobacter* sp.) or of bacteria highly efficient in the transformation of organic phosphorus to inorganic phosphorus (*Bacillus megaterium*), negative results predominate in the literature. A common explanation for stimulation of crop yields by soil inoculation with either of these organisms (i.e. the reported successes of soil inoculation) is that sufficient population densities of the alien organism were added to the soil that the death and decay of their biomass significantly increased soil-fixed nitrogen or phosphorus reserves.

Even with the scattered successes regarding generalized soil inoculation, the preceding evaluation of microbial interactions in soil suggests an explanation for why colonization by alien organisms should not be expected. Soil is a complex ecosystem in which a vast array of microbial populations have developed. It is reasonable to assume that in a mature (climax) system, if a niche exists for a microbe to function, organisms will most probably already have been selected to fill it. The occupant of the niche would be the most efficient organism to fulfill that function. Thus, to alter the composition of the soil microbial community through inoculation, the added organism must be able to survive in the ecosystem sufficiently long to become established and either (i) to fill a niche that is not occupied in the soil ecosystem, or (ii) to be capable of dislodging preexisting populations.

These prerequisites underscore the situations where soil inoculation appears or has been documented to be successful. In most cases, a new niche is created in the soil for the inoculated organism to occupy. This is seen in inoculation of root

tissue with *Rhizobium* strains (the new niche is provided by the invasion of the nascent root tissue), addition of nitrogen-fixing blue-green algae to rice paddy fields (the new niche is provided by flooding of a previously drained soil), or addition of a specialized degrader population to a site to be renovated (the new niche is provided for the microbes capable of degrading the pollutant). In the latter situation, the probability of enhanced pollutant mineralization due to inoculation still rests partially on the capability of the amended organism to outcompete any indigenous populations with similar metabolic capabilities. Future expansion of the lists of inoculation success will be predicated upon better prediction of the nature of the unoccupied niche in soil and an improved understanding of phenotypic microbial traits that allows for augmented capacity to compete with indigenous microbes, thereby usurping their position in the soil community.

7.6 Concluding Comments: Implications of Soil Microbial Interactions

It is reasonable that the vast majority of soil microbiologically oriented projects have involved evaluation of the native soil procaryotic populations and, to some degree, fungal activity in isolation from consideration of interactions with other soil populations. This emphasis is understandable since the primary organisms responsible for decomposition of native biomass and xenobiotic compounds are bacteria, fungi, and, to some degree, actinomycetes. Yet, it is mandatory to realize that the rate of decomposition processes in soil is controlled by the interactions of these primary feeders with a vast array of secondary feeders. The indirect activities of mixing of organic debris with soil, inoculation of this biomass with degrader populations, and the mincing of large plant structures have a significant and obvious effect on decomposition rates. The rate is further controlled by the feeding activity of parasites and predators. Thus, any evaluation of ecosystem dynamics must not only include consideration of the metabolic capacity of the soil microbial community and their physical and chemical needs but also must be conducted with an appreciation of the impact of higher organisms on the vitality and survival of these front-line organic matter mineralizers.

Where to next? This analysis provides a reasonable depiction of the breadth of interactions between soil microbes. Adaptation of the microbial community to the soil physical and chemical properties along with the various microbial interactions yields optimally functioning, sustainable ecosystems. The biological components, ranging from viruses to higher plants and animals, not only populate soils but also interact in a manner that optimizes ecosystem function and sustainability, frequently under conditions that could be easily interpretated as life limiting

or threatening. The diversity of life in soil is quite impressive, even within soil systems that could easily be concluded to be adverse to biological activity, such as arid desert soils. Even though the size of the living portion of soils is certainly quite variable (consider different energy inputs and moisture limitations between a desert system and a forest), the complexity of the microbial community provides "food for thought" for considerations of how the individual microbial populations compete and survive in the complex soil matrix. Thus, data such as the following for population densities of microbes/g soil only provde an example of the large population densitities of microbes that could be anticipated to be found in soil. Populations g^{-1} dry soil as large as 10^{12} bacteria, 10^4 nematodes, and 25 km of fungal mycelia are commonly cited (for example, see Young and Crawford 2004). Several thousand different bacterial species are commonly said to occur in a single gram of soil yet the surface area of the soil occupied by the soil microbes is said to be only 10^{-6}% (Young and Crawford 2004). Even without considering the complexities of adding higher plants and animals to our deliberations, apparent discrepancies between high population densities and microbial diversities with low occupancy of the soil particle surface area provide impetus for a more detailed examination of the details of microbial habitats within the soil matrix.

Further support for this reexamination of the life factors controlling microbial diversity is derived from situations where observations of actual infield interactions delimiting microbial community development differ from what would be predicted by the processes evaluated in this chapter. Such situations demand further research to determine if our concepts are in error or if they are simply incomplete. For example, data presented earlier in this chapter document the rapid decline of *E. coli* populations when laboratory cultures of the microbes are amended to a field soil. It is easy to accept this observation as being consistent with our understanding of soil structure and function since it is generally accepted that the survival traits of the fecal coliform would be those associated with growth and development in the intestinal environment (a nutrient-rich site with controlled temperature, pH, etc.). To survive and become a contributing member of the soil microbial community, the microbe would need to develop the capacity to survive within the biologically, physically, and chemically stressful soil microsite. In the soil environment, the capacity of *E. coli* to compete with the better adapted soil microbes can be concluded to be limited. Case closed ... not quite! DePas et al. (2014) found that this enteric bacterium was protected by the physical structure of its microenvironment from desiccation and chemical toxicity as well as from the predatory bacterium *Myxococcus xanthus* and the nematode *Caenorhabditis elegans*. These authors demonstrated this potential protection by growing the enteric bacterium in media that supported biofilm production. That is, inclusion of the *E. coli* in biofilms protected it from predation.

A further observation that supports the need to consider the importance of biofilms in controlling microbial growth and survival in soil is the fact that the microbial contributions to biofilm development seem to be genetically controlled. That is, genes within the bacteria are required for biofilm formation and function, including both quorum sensing processes as well as synthesis of the extracellular polymeric substances supporting the biofilm physical structure (see Huang et al. 2003, Seaton et al. 2013, and Vivant et al. 2015 for examples of such studies).

These observations support the conclusion that biofilm formation and quorum sensing may contribute to the development and survival of a diverse microbial community (for example, see Wang et al. 2014, Hinsa-Leasure et al. 2013, and Redmile-Gordon et al. 2014). Along with the potential for enhanced microbial survival in soil, data also suggest that biofilms have a possible role in movement of bacteria within the soil matrix. Warmink and van Elsas (2009) demonstrated that biofilm formation at the fungal hyphal tip facilitated translocation and growth of the bacteria. These observations alone support the conclusion that more research is justified to elucidate the full role of soil biofilms in selection of the active microbial community members, controlling the interaction between cells, and the optimization of the microbial function. It is not too difficult to conceptualize a small bacterial coloney residing on a soil aggregate being consumed by protozoa or nematodes, especially considering that the colony need not consist of more than perhaps a few hundred cells.

But this somewhat simplistic view is complicated by the array of micro- and macropores within which the biofilm would develop. As noted previously, competition for space can become a significant controller of microbial growth in soil. This limitation would not only affect the size of the population of microbes in the biofilm but would also necessarily, due to the presence of the smaller biofilm size, limit the diversity of the microbes composing it. Thus, elucidation of the limiters of biofilm development and function must include consideration of the soil pore structure. Answers to questions such as "How large can a biofilm become before the soils become plugged by microbial cell mass?", "How does the deformation of the pore structure by biofilm mass affect transport of nutrients and products to and from the cells in the biofilm?", and "What is the extent of the impact of soil pore structure on the microbial metabolic reaction kinetics?" become pertinent to explaining the factors controlling microbial community development in soil systems.

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8

The Rhizosphere/Mycorrhizosphere

The soil microbial community is driven by solar power. The primary energy supply is photosynthetically fixed carbon which enters the soil ecosystem primarily as plant biomass and exudates. Hence, it is logical that a site of maximized biological activity is the portion of the ecosystem encompassed by the soil and root interface – the rhizosphere. The provision of energy to the microbial community by root exudates, dead roots, and sloughed cells results in intense microbial metabolic activity plus an enhancement of microbial interactions – e.g. competition, symbiosis, amensalism, and predation – over levels occurring in nonrhizosphere soil. Furthermore, since plants benefit from the mineralization capacity of the soil microbial community and the improved soil structure derived from the enhanced microbial activity, the rhizosphere microbial community represents that portion of the soil ecosystem with maximal effect on the aboveground community.

During the growing season, the rhizosphere is an expanding focus of biological energy. New microbial habitats are produced continually through root growth. Thus, scientific interest in elucidation of rhizosphere processes is derived from the desire to understand the basic biological activities occurring therein as well as from the fact that rhizosphere interactions provide a model for successful modification of native microbial community dynamics (e.g. root inoculation with symbiotic nitrogen-fixing populations) as well as for study of the potential uses of genetically modified microbes. Opportunities for use of genetically modified microorganisms in the rhizosphere and adjacent soils include population manipulation to reduce problems with plant pathogenic microbes as well as to achieve soil renovation or other bioremediation objectives.

Therefore, this analysis of rhizosphere/mycorrhizosphere properties is presented with the overall objective of elucidation of the properties of the microbial processes occurring within the rhizosphere and those factors which make it an unique portion of the soil ecosystem. Traits of the total rhizosphere will be examined along with the microbe–plant interactions affecting plant growth and

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microbial community development. This analysis will be followed by an evaluation of mycorrhizal symbioses and the impact of these associations on properties of the rhizosphere.

This analysis of rhizosphere/mycorrhizosphere properties is presented with the overall objective of elucidation of the properties of the microbes and the soil itself that control overall ecosystem function and sustainability. Such research requires accurate determination of rates and location of the biological processes. As you consider the limitations to quantification of the microbial community in comparison to plant growth, the questions of how the reaction rate changes with distance from the root and what controls the magnitude and rate of the biological reactions become more complex. That is, "Is the soil sampled at 0.5 cm from the root or is the soil collected at 1.0 or 2.0 cm more representative to the soil system itself?"

To provide a clear understanding of soil sample collection limitations, the research presented in Chapter 8 represents classical interpretation of experimental field data. The more modern methods are presented in chapters of the text based on modern methods such as DNA analyses, statistical variations in soil sampling and management, etc. (for example, the nitrogen cycle presentations). Grouping of the research data into that developed with more classical procedures (Chapter 8) and a separate group that includes the more current analytical methods (DNA analyses) will hopefully allow for better understanding of soil biological processes. Note that the References section for this chapter is heavily weighted in what could be said to be the classical class whereas the more current DNA-based studies are presented in large part in the newer publications reported throughout the chapter.

8.1 The Rhizosphere

By definition, the *rhizosphere* is that portion of the soil under the direct influence of the roots of higher plants whereas the *rhizoplane* encompasses the root surface and its adhering soil. As shown in Figure 8.1, the soil microbes in the vicinity of the growing roots are stimulated by the provision of a surface upon which to grow as well as by the nutrients contained in leachates, sloughed cells, and decaying roots. Microbial colonies develop unevenly along the root surface, with maximal populations occurring where the exudates are lost from the root. Therefore, occurrence of microcolonies is a good indicator of carbon and energy source leakage from the root.

Characteristics that define the rhizosphere are as follows.

- The properties of the plant are the predominant controllers of the ecosystem rather than those of the soil. This situation results from the fact that the plant is

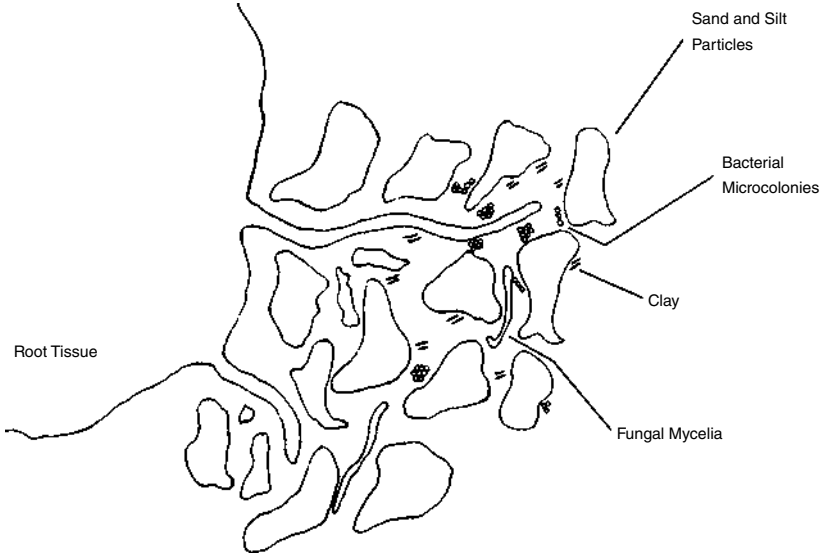

Figure 8.1 Schematic diagram of the physical relationship of primary components of the rhizosphere (not drawn to scale).

supplying the nutrient and energy source, plus space for the microbes to grow, as well as altering the gaseous regime of the soil.

- The microorganisms prevailing in the rhizosphere originate in the soil and are characteristic of the soil microbial community. But because of the variation in the nature of root exudates between plant species, the specific organisms comprising the rhizosphere microbial community do vary with plant species and soil type (e.g. Grayston et al. 1998; Latour et al. 1996; Maloney et al. 1997).
- Because of the combination of increased microbial respiration occurring in the root zone compared to the adjacent nonrhizosphere soil and the root respiration, the gas regime around the root differs from that of the surrounding soil. Carbon dioxide levels are generally elevated and molecular oxygen tensions are reduced in comparison with the gaseous content of nonrhizosphere-impacted soil.
- The influx of carbon and energy in the form of root exudates selects for microbes capable of using the nutrients most efficiently – the fast-growing microbes. In comparison, nonrhizosphere microbial communities typically exist in a "feast and famine" state. Nutrients provided to nonrhizosphere populations primarily result from the influx of nutrients contained in infiltrated water (a typical source in a nonmanaged ecosystem is fixed carbon mobilized by the decay of surface litter) or are carried into the site through movement of animal life (including the nutrients contained in the animal body when the organism expires as well as organic substances translocated by the activity of ants, termites, and earthworms).

It is tempting to consider the rhizosphere community to be continuously bathed in nutrients. This concept may be of value in developing an overall model of the community, but it is far from the situation at the microsite. As is discussed in greater detail later in this chapter, quantities of root exudates as well as their chemical composition vary along the root length, change throughout the growing season, and are dependent upon root density and plant–plant interactions.

- Since many of the products of root metabolism are acids (including carbon dioxide which forms a weak acid when dissolved in soil water), soil minerals are solubolized in the rhizosphere (e.g. Bolan et al. 1997; Hinsinger 1997; Krishnamurti et al. 1997). Thus, osmotic impacts on microbial growth are accentuated in the rhizosphere, mineral nutrient availability is modified, and clay mineral transformation may occur. For example, linkage of potassium availability and loss of potassium ion from mica flakes plus transformation of phylogopite into vermiculite in inoculated pine root (*Pinus sylvestris* L.) rhizosphere soils were shown by Leyval and Verthelin (1991).

8.1.1 The Microbial Community

The rhizosphere effect or the degree of stimulation of an activity of population by the energy input of the plant root has classically been described by evaluation of the ratio of activity per unit weight of rhizosphere soil to the activity per unit weight of nonrhizosphere soil (the R/S value). Values greater than 1 indicate selective stimulation in the rhizosphere, equivalence of the two activities suggests no rhizosphere effect, whereas a R/S ratio of less than 1 reveals inhibition of the activity in the rhizosphere.

Rhizosphere bacterial populations: Bacterial populations are stimulated by root exudates. Populations greater than $10^9 g^{-1}$ rhizosphere soil are commonly detected. Selective stimulation of rapidly growing, gram-negative, rod-shaped bacteria (*Pseudomonas* sp., *Flavobacterium* sp., and *Alcaligenes* sp. especially and occasionally *Agrobacterium* sp.) is observed. These bacterial species are found as microcolonies covering approximately 4–10% of the root surface. Competition, the primary selective, intermicrobial interaction, is based on growth rate, metabolic versatility, and growth factor requirements as follows.

- *Growth rate*: The conditions of the rhizosphere favor those bacteria which have short generation times. Clearly, organisms that convert the carbon and energy contained in the root exudates to biomass efficiently and rapidly will achieve greater population densities and occupy more of the space than the less vigorous and efficient members of the community.
- *Nutrients*: A variety of fixed carbon compounds are available to the microorganism within the rhizosphere. Stress of competition may result in exhaustion of a

particular growth substrate, whereas abundant supplies of others remain. Thus, successful bacterial populations are those capable of using a variety of biochemical growth substrates. Data suggest that, in general, the organisms in the rhizosphere are not carbon limited (e.g. Cheng 1996), but supplies of specific carbon sources may become more limiting due to intense competition.

- *Growth factors*: Root exudates commonly contain a variety of amino acids and vitamins which may be used as microbial nutrients or growth factors. Comparison of the growth factor-synthesizing capacities of rhizosphere bacteria suggests that some advantage is conferred on organisms requiring amino acids and no other growth factors. Interestingly, the proportion of bacteria in the rhizosphere which require complex nutritional growth factors declines, but their actual numbers increases. This somewhat confusing observation results from the fact that the number of these organisms in the ecosystem increases but the total population increases at an even faster rate. Therefore, although there are more growth factor-requiring bacteria in the rhizosphere than are found in nonrhizosphere soil, they make up a smaller portion of the total population.

Two other factors affecting microbial growth in the rhizosphere are surface area available for colonial development and antibiotic production. Since the simple addition of nutrients to soil can also stimulate the same group of bacteria as are stimulated in the rhizosphere, nutrient supply has to be a primary selective factor in the rhizosphere but, as nutrient needs are met, the point of competition may be shifted to factors impacted by the minute distances between individual cells and physical obstructions to growth, e.g. surface area and toxicant production. Competition for surface area affects population development primarily at the microsite where cell–cell interactions are intensified. As indicated in Chapter 7, antibiotic production appears to play a role in ammensal interactions in the rhizosphere. With the close proximity of bacteria, the intense competitions occurring, and the positive impact of inoculation of plant roots with antibiotic-producing microbial strains, antibiotics could be of major importance in this ecosystem, but absolute elucidation of antibiotic production and importance in the root ecosystem has yet to be developed.

Fungi in the rhizosphere: Quantification of the rhizosphere effect on soil fungal populations is equivocal at best. This difficulty is derived, to a large degree, from inability to separate active from inactive fungal propagules. Fungal population densities may be quantified through enumeration of colonial development on defined media or mycelial strands may be counted directly in soil samples. Colonies growing on defined media may result from outgrowth of spores contained in the soil sample or from mycelial strands. Direct observation of hyphal strands in soil provides a measure of total fungal biomass, but not species or genus identity. Colonies growing on the defined media can readily be classified to genus and species, but they may

have developed from a quiescent spore serendipitously existing in the environs of the root or from an actively respiring fungal colony. Thus, data collected for the evaluation of rhizosphere effect on fungal populations are difficult to interpret. Total fungal counts appear not to be impacted by association with the root, but some individual populations may be stimulated. Plant roots do produce compounds which stimulate outgrowth of fungal resting structures and hyphal development. Similarly, zoospores of phycomycetes are chemotactically attracted to the growing root tip. These observations suggest a positive rhizosphere effect on fungal populations.

Other rhizosphere inhabitants: Organisms whose populations tend not to react to influxes of readily decomposable organic matter are usually not affected by root growth. This grouping includes actinomycetes, protozoa, and algal populations. The actinomycetes generally derive their energy supply from decomposition of less readily decomposable soil organic matter components whereas algal populations use solar energy. Protozoan populations are limited by the distribution and density of prey populations. Because of the high population density of prey required to support increases in protozoan cell numbers and the limited precision of the methods used to quantify protozoa in soil, a slight increase of protozoa in rhizosphere soil could occur and escape detection.

8.1.2 Sampling Rhizosphere Soil

The basic definition of the rhizosphere (that portion of the soil profile under the direct influence of the plant root) provides an excellent conceptual resolution of the rhizosphere and nonrhizosphere domains but, from a practical viewpoint, it provides little upon which to base a soil sampling procedure. Questions which may arise in preparation of rhizosphere soil samples pertain to the distance from the root that the plant effect extends, the degree of the impact (for indeed, we realize that there are minute changes in soil properties at considerable distance from the growing root resulting from the presence of the root), and how to avoid diluting true rhizosphere soil with nonrhizosphere soil.

Even if the effect in question will extend hypothetically only 1 cm from the root surface, that region of impact of the root exudates is not uniform along the length of the root. Greater intensity of interaction of the root with the soil community is found at the zone of active growth. Therefore, defining the rhizosphere as extending 1 cm from the root surface in all directions for the full extent of the root length will still necessarily result in dilution of more active soil with less active soil. Since in reality the capacity to answer these questions involving the definition of the exact portion of the soil mass impacted by the root is nonexistent, most studies of the rhizosphere have involved an arbitrary definition of the soil mass to be collected for field soils, in particular, and a variety of means of physical separation of roots and soil in laboratory and greenhouse studies.

The arbitrary nature of collection of rhizosphere soils in field plots is demonstrated by an examination of common soil sampling techniques. The more time-consuming method is to dig a soil pit around a plant and aseptically remove soil within a predetermined distance of the plant root – commonly a 1 cm distance. For example, Ruark et al. (1991) compared the interaction of acid rain and ozone treatments on hydrogen, aluminum, calcium, and magnesium ion concentrations in root-adjacent (rhizosphere) and bulk (nonrhizosphere) soils for loblolly pine (*Pinus taeda* L.) seedlings growing in the field by this type of method. The procedure was adequate to demonstrate gross differences in anion concentration due to atmosphere problems and plant growth. Alternatively, the plant may be carefully removed from the soil. Nonadhering soil is allowed to fall to the ground whereas soil adhering to the root system, defined to be the rhizosphere soil, is gently shaken into a collection container. Soil in direct contact with the root that cannot be gently shaken from the root surface, but must be washed from the root to be collected, is arbitrarily defined as rhizoplane soil. The limitation associated with either of these procedures is that root-impacted and unaffected soils are inevitably mixed at unknown ratios. Thus, rhizosphere effects are at least underestimated and potentially may be totally overlooked should sufficient dilution with unaffected soil occur.

Techniques similar to those used to separate nonrhizosphere and rhizosphere soils in the field may be applied to microcosm studies incubated in the laboratory or greenhouse. For example, see Norton and Firestone (1991) where soil was sampled at small intervals from coarse, and fine young roots of Ponderosa pine (*Pinus ponderosa* Laws.) seedlings for analysis of the metabolic status of the bacterial and fungal communities. Alternatively, rhizosphere and nonrhizosphere soils may be physically isolated in the microcosm (e.g. Hartel et al. 1989; Martens 1982). Tate et al. (1991) isolated pitch pine (*Pinus rigida* Miller) seedling rhizosphere soils from nonrhizosphere soils with a 1 μm mesh fabric. Soluble exudates could pass through the membrane, but root tissue was retained. With either procedure, limitations of dilution of plant-impacted soil by nonplant-affected soil compromise data interpretation as was indicated for field soil sampling procedures.

It is reasonable to assume that results from the study of bulked soils underestimate the true stimulatory (or even inhibitory) effect of the plant root on soil microorganisms. This leveling of the data is further accentuated when the distribution of the active microbes along the root is considered (recall that root exudates are not released continuously along the root, hence microbial colonies are found in "hot spots" in the rhizosphere). Thus, the bulked rhizosphere soil sample is not only diluted by nonrhizosphere soil but it is also a composite of microsites containing highly active microbial communities plus microsites in which a more reduced stimulation or inhibition soil microbial activity has occurred.

8.1.3 Plant Contributions to the Rhizosphere Ecosystem

The role of the plant in the rhizosphere is to provide a surface for microcolony development and to produce the fixed carbon compounds used by soil microbes for carbon, energy, and as growth factors. Major questions regarding this process are associated with quantification of the quantities of photosynthate entering the rhizosphere, identification of the organic components of root exudates, and elucidation of the variation in these parameters throughout the growing season and between plant species. A related concern in native systems and with dual cropping systems is determination of the impact of the interaction of root systems from different plant species on the quantities and composition of root exudates.

As a result of the observation that in native soils, photosynthate produced by higher plants (as compared to more limited quantities of carbon fixed by algal activity) generally provides the primary energy source for the soil microbial community, there has been a long-standing interest in quantifying the proportion of carbon fixed by the plant released into the rhizosphere community and the ultimate deposition of the fixed carbon (i.e. incorporation into humic substances, microbial biomass, or oxidation to carbon dioxide by the soil biota). Microbiologists see this carbon/energy source emanating from the plant root structure as the major driving force for the soil ecosystem. In contrast, plant biologists may regard production of root exudates as both a benefit to the plant and a potential growth limitation. Benefits are accrued from the stimulation of soil microbial populations whose functions encourage plant development (e.g. through nitrogen mineralization, reduction of plant pathogenic interactions, and synthesis of growth-controlling plant hormones such as indolacetic acid and giberellic acid). Negative implications are associated with the potential for excessive loss of photosynthate and therefore carbon and energy available for plant biomass production.

A variety of means of quantifying and tracing the movement of root exudates have been developed. Logically, it would seem that a useful procedure for assessing the nature and quantity of fixed carbon products exuded from growing roots would be to quantify the biochemicals accumulated in the growth medium of plants grown under axenic conditions in liquid culture either in a greenhouse or growth chamber culture (e.g. see study conducted by Griffin et al. 1977). The primary difficulty encountered with such studies is that the microbial community has a stimulatory effect on root exudate production and may alter the nature of the biochemicals contained in root exudates (Barber and Martin 1977; Klein et al. 1988). Barber and Martin (1977) found that between 5% and 10% of the photosynthetically fixed carbon in wheat and barley plants was lost from roots grown under axenic conditions whereas 12–18% of photosynthate was lost from roots growing in nonsterile soils. Thus, realistic studies of root exudation should be conducted in situations reflecting native soil conditions, as closely as possible.

Utilization of radio-labeled carbon with plants growing in soils appears to provide an improved estimate of photosynthate incorporation into soil fractions. For this, root exudates are evaluated in greenhouse or growth chamber experiments where photosynthate can be labeled with ^{14}C-labeled carbon dioxide. Aboveground and belowground portions of the plant must be physically separated so that access of the soil microbial community to $^{14}CO_2$- or ^{14}C-labeled photosynthetically produced organic compounds can only occur through the root tissue. Movement of the ^{14}C-labeled photosynthate into the rhizosphere is determined by quantifying the $^{14}CO_2$ evolved from rhizosphere soil and the incorporation of ^{14}C into humic substances (e.g. using sodium hydroxide fractionation of soil organic matter) and microbial biomass (e.g. with the chloroform fumigation technique). An alternative to using ^{14}C-labeled substrates for quantifying root exudate production was exploited by Barber and Lynch (1977) who utilized the relationship between the quantity of nutrients available for microbial growth and the mass of cells synthesized (i.e. growth yields). By calibrating rhizosphere microbial biomass produced per unit of growth substrate, a growth yield constant was developed. This constant was used to convert the quantities of microbial biomass produced in the presence of roots growing in liquid culture in growth chambers into mass of photosynthate available for their growth.

A wide range of values for root exudate production are reported in the literature. Nutrients available to rhizosphere microbes vary with plant species, plant developmental stage, and soil temperature, as well as species distribution of the plant community. Newman (1985) concluded that soluble root exudate ranges from 1 to 10 g per 100 g dry roots. Values found in the literature vary depending upon the inclusiveness of the data collected. Some reports of plant carbon incorporated into soil fractions include total plant carbon retained in soil organic fractions whereas in other studies, root exudate production is evaluated exclusive of carbon contributions by root cell death and root death. Martin (1977) recorded a range of rhizodeposition of 14.3–44.4% of photosynthetically fixed carbon (for wheat plants) whereas Davenport and Thomas (1988) found 10% of $^{14}CO_2$ fixed by corn (*Zea mays* L.) in belowground plant components compared to 40% for bromegrass (*Bromus inermis* Leyss). This differential separation of fixed carbon between aboveground and belowground plant biomass resulted in a rhizodeposition rate in the bromegrass twice that of the corn. During the last 31 days of the 55-day period from germination to seed set of blue grama (*Bouteloua gracilis* [H.B.K.] Lag.), 33% of the fixed carbon was found in the root biomass, 23% in the root-derived organic matter contained in the soil, and 22% was released as carbon dioxide (Dormaar and Sauerbeck 1983). These values contrast to early studies such as that by Martin (1977) where 0.8–1.3% of total organic carbon in root-free soils had been derived from plant photosynthate.

The rhizosphere microbial community can increase the rhizodeposition rate of photosynthetically fixed carbon. Microbes significantly increased root exudation of *Agropyron cristatum* and *Agropyron smithii* but had no effect on *B. gracilis* (Biondini et al. 1988). For the former grass strains, root exudates were reduced 40% and 83% respectively from native soil values when grown in axenic culture. The root biomass of *B. gracilis* was increased by the presence of an active rhizosphere community (Klein et al. 1988). This microbial stimulation of root exudation may result directly from synthesis of plant hormones by the microbes that are stimulatory to exudate production. An indirect stimulation of exudation could result from microbial utilization of the exudates, thereby preventing their accumulation. This would increase outward diffusion of exudate components from the root.

Merckx et al. (1986) compared the release of photosynthate by maize and wheat roots growing in a growth chamber. After six weeks growth, 1.5% and 2.0% of the corn and wheat photosynthate, respectively, was found as soil organic carbon residues. Photosynthetically fixed carbon dioxide was also detected as soil microbial biomass and as carbon dioxide respired by the soil microbial community. For the wheat plants, approximately 20% of the fixed carbon was detected in each of these carbon pools. Distribution of photosynthetically fixed carbon within these various pools can vary with soil temperature (Meharg and Killham 1989). Increasing the soil temperature from 5 °C to 25 °C increased root-soil respiration from 5.7% to 24.15%. The proportion of the photosynthate retained in the plant root and soil was greater at 5 °C and 25 °C, with a minimum at 15 °C. The total fixed carbon released as root exudates by *A. cristatum*, *A. smithii*, and *B. gracilis* was estimated to be 8%, 17%, and 15% of photosynthetically fixed carbon.

Microbial growth in the rhizosphere can also be affected indirectly by the quantities of plant nutrients available for plant biomass production. Merckx et al. (1987) grew maize plants at high and low nutrient levels. Plant biomass production was limited at the low nutrient levels after 35 days growth. In soils with the lower nutrient level, 2% of the total $^{14}CO_2$ fixed by the plant was retained in the soil at all harvest times (38, 35, and 42 days) whereas with the nongrowth-limiting nutrient levels, 4% of the plant photosynthate was retained in the soil. Maximal soil incorporation was found in the lower nutrient soils at 35 days growth whereas the quantities retained in the high nutrient soils increased throughout the study period. Microbial biomass contained between 28% and 41% of the total soil ^{14}C in the lower nutrient soil whereas in the higher nutrient soil, this value ranged from 20% to 30% of soil residual photosynthetically fixed carbon.

When evaluating plant contributions to soil microbial energy transformation, it is important to consider annual turnover of the root biomass itself. This fixed carbon contribution to microbial nutrient cycling may be considerable. For example, Whitford et al. (1988) found that in the Chihuahuan Desert, 85–90% of the mass of herbaceous annual roots and 40% of the mass of woody shrub roots was decomposed

annually. These rates are comparable to or greater than similar processes reported for mesic ecosystems. For comparison, Dahlman and Kucera (1965) found an annual increment in the surface 25 cm of soil (the A horizon) of a prairie grass land in central Missouri of 429 g m^{-2}. This represented about 25% of the total root biomass in this horizon, suggesting an annual root biomass turnover rate of about 25%. For comparison, in a study of live root decomposition in four forests (a mixed deciduous forest in Virginia, a mixed deciduous forest and a *Pinus resinosa* plantation in Massachusetts, and an *Acer saccharum-Quercus borealis* forest in Wisconsin), losses of between 20% and 60% of their initial mass over a 4-year period were detected for each forest (McClaugherty et al. 1984).

Other methods used to trace movement of carbon fixed by the plants through the soil microbial and organic matter pools include pulse-chase experiments with $^{14}CO_2$ (e.g. Rattray et al. 1995) and experiments assessing ^{13}C abundance in soils converted from growth of C-3 vegetation to C-4 plants (e.g. Qian and Doran 1996). With the former studies (pulse-chase), plants are incubated in a $^{14}CO_2$-enriched atmosphere. After an appropriate incubation period in the enriched atmosphere, the ^{14}C-enriched air is replaced with normal ambient air. The change in the ^{14}C enrichment of plant tissue and soil organic components is quantified with time. Initially, the bulk of the radioactivity is detected in plant tissue. After the labeled carbon dioxide is removed from the atmosphere, no further increase in labeled plant tissue occurs. Then, movement of the labeled carbon into soil components is quantified. These studies provide an indication of the amount of plant carbon lost in root exudates and the time course of the process. The use of the stable, heavy isotope of carbon (^{13}C) is valuable in situations where plant communities can be changed (switched from C-3 to C-4 plants) and minimal alteration of soil structural properties is desired. In this case, the increased incorporation of ^{13}C into biomass of the C-4 plants provides the tracer for carbon movement in the soil system.

The composition of root exudate is a primary parameter in selecting for individual species active in the rhizosphere community and the diversity of that community. The composition of root exudates varies with growth condition of the plant and its developmental stage. The variety of compounds which may be contained in root exudates is indicated by a list of candidates compiled by Alexander (1977). These soluble, organic carbon sources accumulated by plants grown aseptically include the following.

- *Amino acids*: all naturally occurring amino acids.
- *Organic acids*: acetic, butyric, citric, fumaric, glycolic, lactic, malic, oxalic, propionic, succinic, tartaric, and valeric acids.
- *Pentoses and hexoses*: arabinose, deoxyribose, fructose, galactose, glucose, maltose, mannose, raffinose, rhamnose, ribose, sucrose, and xylose.
- *Pyrimidines and puridines*: adenine, cytidine, guanine and uridine

- *Vitamins*: *p*-aminobenzoate, biotin, choline, inositol, nicotinic acid, pantothenate, pyridoxine, and thiamine.
- *Enzymes*: amylase, invertase, phosphate, and protease

This smorgasbord of readily catabolized organic substances is supplemented by all components contained in sloughed root cells and decaying root biomass. As was indicated above, as much as one-fifth to one-third of the fine root biomass may turn over annually. The selective stimulation of microbial populations by the root exudates is readily demonstrated by the alteration of metabolic diversity (see Chapter 2 for a description of methods for determining the soil community property) compared to nonrhizosphere soil (e.g. see Garland 1996a, 1996b for use with whole soils and Frey et al. 1997 for study of soil isolates). The method has been useful in assessing the effect of changes in root exudates due to elevated atmospheric carbon dioxide levels on rhizosphere community dynamics (e.g. Hodge et al. 1998; Rillig et al. 1997).

The activity of the rhizosphere population is determined not only by the composition of root exudate, sloughed cells, and decaying root tissue but also by the total quantities of fixed carbon produced, as was suggested above. From considering the relationship of these three fixed carbon pools to total plant productivity, it is clear that rhizosphere activity is dependent upon plant nutrient status and reaction of the plant to environmental stresses. Examples of data reflective of this microbe–plant growth state interaction are provided by studies of ryegrass (*Lolium perenne*) under a variety of fertilization strategies (Turner and Newman 1984; Turner et al. 1985), and in studies of grassland management (Ingram et al. 1985). The ryegrass studies demonstrated increased root exudation under phosphorus deprivation. Evaluation of range plants showed an interaction between herbage removal and irrigation, with irrigation causing significant declines in general rhizosphere activity which was mitigated by herbage removal. Active fungal hyphae were decreased by both treatments whereas overall microbial activity was stimulated by herbage removal, suggesting a shift in the relationship of active fungal and bacterial populations in the stressed plant rhizosphere.

A further question regarding the rhizosphere effect relates to the longevity of the plant impact on the soil community following demise of the plant. Death of the plant results in a decline in the rhizosphere community. Little, if any, microbiological effect is carried over in the soil until the next invasion by root tissue. Although there is an impact of adjacent plants on the nature of the rhizosphere community, preceding plant species existent in the ecosystem have little effect on rhizosphere community composition of succeeding plants. The new vegetation determines, to a large degree, its own rhizosphere community composition. Exceptions to this statement of no carryover of rhizosphere effects relate to soil properties which are altered by the presence of root surface or root modulating organisms. For example, fixed nitrogen accumulated in a soil environment due to

the symbiotic association of *Rhizobium* sp. and legumes may provide a nitrogen nutrient source for succeeding plant communities. Similarly, physical or chemical modifications of the soil mineral environment necessarily persist.

8.1.4 Benefits to Plants Resulting from Rhizosphere Populations

Development of an active rhizosphere community has a variety of indirect and direct impacts on plant biomass production. Many of these benefits are proposed since it is difficult to quantify minute modifications of the plant root environment and their effect on overall plant development, but logic combined with sound data suggest that an active, dynamic rhizosphere microbial population is beneficial, if not essential, for optimal plant productivity. Rhizosphere property modifications beneficial to plant growth and development range from the more difficult to prove production of growth-promoting substances to the clearly documented gains of symbiotic root associations involved in nitrogen fixation.

A wide variety of rhizosphere microorganisms have been demonstrated to produce plant growth hormones, including indolacetic acid (IAA), gibberellins, and cytokinins (e.g. see Nietko and Frankenberg 1989; Tien et al. 1979). These substances could be proposed to stimulate root tissue development, thereby increasing the capacity of the root system to provide nutrients and water required for aboveground biomass function.

A more indirect, but clearly beneficial, result of stimulation of soil community by root invasion is the contribution of rhizosphere microbial communities to development of a stable soil structure conducive to plant community development (i.e. improved soil aggregation) (e.g. Amellal et al. 1998; Andraede et al. 1998) (see Chapter 1 and Tate 1987 for a further discussion of microbial involvement in soil structural development). The rhizosphere community produces polysaccharide material such as capsules and slimes which cement soil mineral particulates into microaggregates. Furthermore, soil structural improvements are gained from fungal mycelial production as well as by association of soil particles with root tissue. This improvement in soil structure through increased soil aggregation results in improvements in soil aeration, water infiltration, and root penetration.

Perhaps the primary benefits of an active rhizosphere community to the plant community result from mineralization of plant biomass. Organic nitrogen, phosphorus, and sulfur compounds are oxidized, thereby liberating ammonium, phosphorus, and sulfate. Thus, the nonbioavailable organic forms of these essential nutrients are converted to plant-available mineral forms.

Two root-based symbiotic associations are instrumental in producing a thriving, stable plant community, nitrogen fixation associations (*Rhizobium*–legume and actinorrhizal associations) (see Chapter 13) and mycorrhizal symbiosis (see

Section 8.2). These interactions increase fixed nitrogen resources and facilitate nutrient transfer to the higher plants.

Additionally, plant growth stimulation may result from solubilization of inorganic plant nutrients by production of organic or inorganic acids by rhizosphere organisms. Microbially produced organic acids may solubolize essential minerals simply through acidification of rhizosphere soil or by chelation (e.g. siderophore production) of the metal. The capacity of organic acids excreted by soil bacteria to solubolize soil minerals is exemplified by studies of 2-ketogluconic acid production by gram-negative bacteria growing in glucose liquid media (Duff et al. 1963). Additionally, the acidification of the rhizosphere environs through metabolic production of hydrogen ions alters the pH sufficiently to mobilize soil minerals (e.g. Gillespie and Pope 1990a, 1990b).

Solubolization of a variety of metals contained in natural and synthetic insoluble salts and minerals has been demonstrated. Siderophores and other metal chelators are synthesized by a variety of bacteria, including common soil organisms (e.g. Carrillo et al. 1992; Fekte et al. 1983; Hoefte et al. 1991; Jurkevitch et al. 1992; Kloepper et al. 1980a; Meyer et al. 1989; Pesmark et al. 1990) as well as mycorrhizal fungal associates (e.g. Cline et al. 1982; Federspiel et al. 1991). A number of examples of stimulation of plant biomass production by these microbially synthesized metal chelators have been published (e.g. Bar-Ness et al. 1991; Crowley et al. 1991; Derylo and Skorupska 1992; Kloepper et al. 1980b). Bossier and Verstraete (1986) have presented a model in which the rhizosphere is the most impacted soil microsite for siderophore synthesis.

8.1.5 Plant Pathogens in the Rhizosphere

A common maxim in plant pathology is that a soil-borne pathogen is more destructive to a plant growing in sterile medium than it would be to a plant developing in native soil where an active rhizosphere community exists. This observation suggests that the rhizosphere microbial community directly or indirectly inhibits invasion of the plant tissue by the pathogen. This disease reduction could result from direct competition between the pathogen and the root microbes, antagonism of the pathogen by root microbes, or alteration of root exudate diffusion into the soil environment in a manner that interferes with chemotaxis of the pathogen to the root. (See van Loon et al. 1998 for a review of this topic.)

Root-inhabiting microbes and plant pathogens may compete for space, nutrients, or even binding sites on the root surface. Space and nutrient competition could result in failure of the pathogen to develop critical population densities for disease initiation whereas competition for specific binding sites would reduce the capability of the plant pathogen to initiate the infection process.

As indicated in Chapter 7, the rhizosphere is one soil site wherein antibiotic production may confer survival advantage upon microbial populations. Both antibiotics

and siderophores produced by a variety of *Pseudomonas* strains have been shown to reduce or preclude infection of wheat roots by take-all disease (Hamdan et al. 1991; Kloepper et al. 1980b; Thomashow et al. 1990; Thomashow and Weller 1990). Other suggested interactions of antibiotic microbes in biocontrol of root disease are associated with *Rhizoctonia* infections (de Freitas and Germida 1991) and onion white rot (Utkhede and Rahe 1980). Similarly, rhizobacteria may also be used to suppress growth of sensitive plants. For example, *Pseudomonas* spp. have been isolated from roots of winter wheat (*Triticum aestivum* L.) and downy brome (*Bromus tectorum* L.) which suppress growth of the downy brome weed.

An additional role of rhizosphere microbes in reducing root disease incidence is interfering with chemotactic attraction of the pathogen to root receptor sites. Chemotactic interactions are more difficult to document in the field but are readily deduced to be of importance in plant disease production. A variety of compounds listed above as components of root exudates may serve as attractants for plant pathogens. Growth of root inhabitants (including mycorrhizal fungi) necessarily reduces both the quantity and diversity of organic compounds diffusing from the root, thereby diminishing the probability of encounter of a plant pathogen.

8.1.6 Manipulation of Rhizosphere Populations

Modification of the composition of any soil community structure through inoculation with exogenous microbes requires a thorough knowledge of microbial interactions as well as "a little bit of luck" if a reasonably predictable outcome of the procedure is desired. Historically, intervention into rhizosphere community dynamics has been a premier example of the microbiologist's expertise – some might say utility. Biological nitrogen fixation has been encouraged through inoculation of legumes with *Rhizobium* sp. to encourage symbiotic nitrogen fixation for many decades (see Chapter 13) Long before the microbiology of the symbiosis was understood, legumes have been exploited in crop rotations for their capacity to increase soil fertility. (Historically, in many agricultural systems, fallow cropping with legumes has been used to maintain soil quality. With these cropping systems, the effect of the legume was maximized by not harvesting the crop. Rather, the crop was plowed under to maximize the quantify of fixed nitrogen and organic matter retained in the system.) The long-established procedure of legume inoculation has been supplemented with methods to supply fungal inocula to stimulate mycorrhizal development plus utilization of a variety of plant growth-stimulating bacteria, including the free-living nitrogen-fixing bacteria of the genus *Azospirillum* (see examples listed in Table 8.1).

Considering that the potential for modification of soil microbial community structure through inoculation with alien strains has long been viewed with pessimism, the question of why root inoculation is frequently successful must be examined. Although as many failures in inoculation for population alteration are

Table 8.1 Examples of reports of modification root communities by amendment with alien microbial populations

Microbe	Recipient	References
Rhizobium spp.	Legume seeds	See Chapter 13
Frankia spp.	Higher plants	See Chapter 13
Mycorrhizal fungi	Higher plants	See text
Pseudomonas putida	Wheat	Vrany et al. (1981)
Bacillus polymyxa	Lodgepole pine	Holl and Chanway (1992)
Pseudomonas fluorescens	Wheat	Parke et al. (1986)
Azospirllium spp.	Spring and winter wheat Maize	Harris et al. (1989) Fallik et al. (1988)
Bacillus sp.	Spring wheat	Chanway et al. (1988)
Azospirillum brasiliense	Wheat	Bashan et al. (1987)
Pseudomonas fluorescens	Potato	Bahme et al. (1988)
Pseudomonas serrata	Wheat and peas	Astrom and Gerhardson (1988)

reported as successes, a cursory review of the literature could lead to the conclusion that the only limitations to the procedure are selection of an appropriate microbial strain for inoculation, development of a carrier to sustain the microbial population, and selection of a delivery system. Indeed, a variety of strains, carriers, and inoculation procedures have been developed; but unanswered basic science questions still remain. Considerations that must be examined include why some inoculation studies are successful while others are not, and why some microbial strains are established in the community more readily than others. Complete understanding of these queries is not available, but some basic information exists to suggest means of improving the probability of successful intervention in rhizosphere community dynamics.

A central maxim in soil ecology is that if a niche exists in an ecosystem for a microbe to function, it will be occupied by a member of the existing soil community. Additionally, displacement of an established, functional member of the community is difficult. Yet, such displacement is frequently desirable in that an improved function of the ecosystem may be achieved by establishment of a more efficient or active microbial strain.

Inoculation of the rhizosphere provides a good example of when this displacement or augmented activity is possible. The first consideration in inoculation of a soil site with an exogenously developed microbial strain relates to the occurrence of a niche in which the microbe has the capacity to function. Nascent root tissue can be a provider of both a habitat and a niche. The external structure of the root is a

surface supportive of colonial development whereas a variety of functional opportunities are derived from the invasion of the root tip into the soil (e.g. decomposition of components of root exudates, nitrogen mineralization, nitrogen fixation) which at least initially have not been implemented by existing populations. Thus, the production of new root tissue overcomes the primary barrier to microbial community composition modification. Remaining obstacles are delivery of an appropriate inoculum to the habitat and the capacity of the alien organism to compete with indigenous populations.

Clearly, inoculation of established root systems is difficult and results in a low probability of development of the inoculated populations. This problem of inoculation of established roots has been overcome by drenching of root systems (e.g. Parke et al. 1986) or it has been avoided entirely by inoculation of seeds before planting. Typically, seeds may be drenched with liquid inocula or pelleted solid carriers of the microbial inoculum prior to planting (see Chapter 13 for discussion of inoculation with rhizobia and Bashan 1986 for use with *Azospirillum* sp.). The assumption underlying this procedure is that the growing root will carry the inoculum with it as the root mass expands. Considering that rhizobial inocula are at least partially established in the field using these methods, seed inoculation may be of less than perfect efficiency, but it still occurs. *Azospirillum brasilense* is also mixed throughout the soil and from plant to plant by growth of wheat roots (Bashan and Levanony 1987, 1989). Alternatives to root drenches and soil inoculation include banding of the microbial inoculum material into the soil adjacent to the expanding root mass. This procedure is underlain by the assumptions that the roots will grow through the band of inoculum, the inoculum will survive sufficiently long in the soil for this encounter to occur, and that the inoculant will compete successfully with indigenous soil microbes for habitable surfaces on the root.

Competition of alien organisms with indigenous soil microbial populations is probably the least understood aspect of any soil community inoculation procedure. The added organisms must be capable of overcoming the normal soil community defenses to invasion as well as coping with soil physical and chemical barriers. Many of the growth requirements for the microbe are met by the provision of nutrients and growth surface by the root; hence dealing with biological competitors becomes the primary difficulty to establishment.

Development of microbial strains which would be useful for field inoculation is generally initiated by isolation of an organism from the indigenous soil population which is amenable for selection of genetic variants. This isolate may then be modified either through selection of natural genetic variants (mutants) or by genetic engineering procedures where appropriate genes are inserted into the genome. In either case, the objective is to develop an organism which is more efficient in a given process (e.g. nitrogen fixation) or which possesses an improved metabolic capacity (e.g. ability to decompose an environmental contaminant).

By starting with a native member of the soil or root community, it may be assumed that most of the necessary traits for successful competition with the indigenous population preexisted in the parent strain. The question then becomes, "Did the genetic manipulation or laboratory culture reduce or eliminate this competitive capacity?" The query is frequently answered negatively. For example, legume nodules are commonly formed by indigenous rhizobial strains in spite of the inoculation of seeds with more efficient varieties. Development of *Rhizobium* strains with improved nitrogen fixation capabilities has become a fine art yet the art is commonly lost when the efficient strains do not enter the site of expression of their enhanced ability – the root nodule. Unfortunately, laboratory-selected rhizobia are not always the best competitors for nodule occupancy (e.g. Klubek et al. 1988; Materon and Hagedorn 1982; Moawad and Bohlool 1984; Robert and Schmidt 1983; Smith and Wollum II 1989; van Rensburg and Strijdom 1985). Variability in nodule occupancy by alien rhizobial strains is exemplified by data from van Rensburg and Strijdom (1985) where nodule occupancy by inoculant strains ranged from 17.7% to 100%, with the highest values associated with legumes growing in soils with limited native rhizobial populations of appropriate specificity for the legume. As discussed in Chapter 13, successful occupancy of the nodule by the inoculant depends upon its capacity to deal with the general rhizosphere population as well as possession of appropriate biochemical traits to efficiently interact with the root to form an effective nodule.

Thus, the rhizosphere microbial community structure has been manipulated through inoculation of the plant with alien organisms to improve plant development (i.e. in most cases, improve crop yields). Great potential exists for further exploitation of these skills to alter the microbial community to accelerate plant community development and reduce soil pollutant loads. Plant growth improvement may be achieved by establishment of members of the rhizosphere community with the capacity to decompose plant toxicants or improve nutrient availability or augment soil granulation whereas the rhizosphere community may also be utilized to couple the nutrient generation ability of higher plants (e.g. fixed carbon supplies) with the ability of a laboratory-generated rhizosphere inoculant bacterial species to degrade a troublesome pollutant (perhaps, even cometabolically) (see Chapter 16).

8.2 Mycorrhizal Associations

Fungus–root or mycorrhizal associations have been known to exist since descriptions by Frank in the early 1880s (cited in Allen 1991). These associations have been extensively described The description, or definition, provided for these fungal and higher plant symbioses, to a large degree, is linked to the definer's bias

toward the importance of the plant or the microbe to ecosystem function. This is best seen by comparing statements by Marx and Allen. Marx (1972) describes mycorrhizal associations as follows.

> The infection of feeder roots of most flowering plants by symbiotic fungi and the transformation of these roots into unique morphological structures called mycorrhizae (fungus-roots) undoubtedly constitutes one of nature's most widespread, persistent, and interesting examples of parasitism. Most plants of economic importance to man are actually dual organisms – part plant and part symbiotic root-inhabiting fungi.

This plant-oriented view stresses the utilization or perhaps even the "theft" of plant photosynthate by the controlled parasites in this symbiotic association. In contrast, Allen (1991) described the fungal–plant interaction from a more neutral or microbially oriented aspect.

> A mycorrhizae is a mutualistic symbiosis between plant and fungus localized in a root or root-like structure in which energy moves primarily from plant to fungus and inorganic resources move from fungus to plant.

In this definition, the mutualistic benefits of the interaction are stressed.

From an environmental or soil community consideration, the fungal–plant associations in mycorrhizal symbioses can be viewed on a gradient of increasing association of the plant and soil community (Table 8.2). At the most extreme position lie the general soil microbial populations. These organisms are involved in mineralization of native soil organic matter, soil-incorporated organic components (plant and animal debris), microbial biomass, and soluble organic carbon components contained in infiltrated water. This community functions totally at the mercy of the nutrient supply which may range from nearly total deprivation (i.e. a desert soil) to absolute luxury (e.g. soil receiving nutrient-laden industrial effluent). Of more direct relevance to the discussion at hand is the fact that the nutrients produced by organic compound mineralization by these general soil microbes may not be directly available to the aboveground community. Clearly, nutrient transfer to higher plants is dependent upon proximity of the microbial colony to active roots. In this case, a locational displacement as small as a few millimeters could determine the rate of mineral nutrient recovery by the plant.

An enhancement of ecosystem three-dimensional associations is derived from the transition from general soil populations to rhizosphere-linked communities. Rhizosphere communities catalyze all the processes described above for nonrhizosphere soil but their value to aboveground biomass sustainability is augmented by improved efficiency of transfer of mineral nutrients to the plant community.

Table 8.2 Comparison of properties of nonrhizosphere, rhizosphere, and mycorrhizosphere soils

Nonrhizosphere	Rhizosphere	Mycorrhizosphere
• All biogeochemical cycles – rates limited by organic matter (humus) availability • Nutrients may be leached to root tissue or groundwaters • Nutrients may be incorporated directly into microbial biomass • Microbial biomass controlled by carbon and energy resources	• Maximal biogeochemical cycling • Nutrients mineralized directly available for plant biomass as well as microbial biomass synthesis • Microbial biomass synthesis controlled by plant productivity and rate of root exudate production	• All biogeochemical cycles are supported – controlled by both plant inputs and soil humus contents • Nutrients incorporated into fungal biomass and transported to plant tissue • Biomass controlled by plant productivity and transfer of photosynthate to the fungus

Note that nonrhizosphere soil metabolic activity is not affected directly by rhizosphere interactions, but mycorrhizal fungi may enter this soil region and catalyze biogeochemical processes.

The ultimate association of soil microbes with growing plants is symbiotic fungal–root associations. This mycorrhizal symbiosis could be said to bridge these community types – nonrhizosphere, rhizosphere, and fungus–root. Although there is an intimate association of the fungus with plant root cells (with some types of mycorrhizae, actual penetration of the root cell itself occurs), the fungal biomass extends well beyond the rhizosphere into nonroot-impacted soil. Thus nutrients may be accumulated from nonrhizosphere soil by the fungus, and transported through the rhizosphere community into the plant root. Inversely, the photosynthate from the plant supports growth and development of the entire hyphal network. Furthermore, this mycelial network may directly link roots of plants of the same or different species in that the same fungal body may infect a number of individual plants.

The physical association of mycorrhizal fungi with plant roots has been extensively described and serves as the primary basis for classification of mycorrhizae. These associations may be classed as ectomycorrhizae, endomycorrhizae, or ectendomycorrhizae. In this grouping of mycorrhizae, the commonly encountered vesicular-arbuscular mycorrhizae are considered endomycorrhizae. A number of reviews of the biology of mycorrhizae have been published (e.g. Allen 1991; Mukerjii et al. 1991). Thus, only basic descriptions of these classes of mycorrhizae will be presented here.

Ectomycorrhizal fungi penetrate intracellularly and partially replace the middle lamellae between cortical cells of feeder roots. These fungi form a dense mycelial

net around and between the plant cells termed a *Hartig net*. Ectomycorrhizal associations are also characterized by a dense, generally continuous hyphal network over the feeder root surface called a *fungal mantle*. This fungal mantle varies from one to two hyphal diameters to as many as 30 or 40 depending upon the fungal associate, the host, and the environmental conditions. Examples of plants forming ectomycorrhizal associations are species in the families *Pinaceae*, *Salicaceae*, *Betulaceae*, and *Fugaceae*. Most ectomycorrhizal fungi are basidomycetes (primarily of the families *Amanitaceae*, *Boletaceae*, *Cortinariaceae*, *Russulaceae*, *Tricholomataceae*, *Rhizopogonaceae*, and *Sclerodermataceae*).

Endomycorrhizae are distinguished by the fact that the fungus penetrates the cortical cells of feeder roots and may form large vesicles and arbuscles (hence the term vesicular-arbuscular mycorrhizae). These fungi do not form dense fungal mantles but do develop a loose, intermittent arrangement of mycelium on the root surface. Endomycorrhizae are formed by most agronomic, horticultural, and ornamental crops, as well as some forest tree species that do not form ectomycorrhizae. The fungal species are phycomyces – many of which are in the genus *Endogone*.

As with many biological classification systems, many organisms form associations that are not easily classified into any of the predominant groupings. For mycorrhizal associations, the *ectendomycorrhizae* constitute this grouping. These mycorrhizae resemble ectomycorrhizae in forming a Hartig net and a fungal mantle. A resemblance with the endomycorrhizae is associated with their penetration of cortical cells. This grouping is the least studied and the nature of the fungal symbionts has not been totally elucidated.

Although mycorrhizae provide intriguing subjects for biological and physiological analysis, the emphasis for this study is to delineate (i) the role of mycorrhizal symbioses in ecosystem function, (ii) their importance in nutrient cycling in soil–plant interactions, and (iii) their utility in bioremediation plans.

8.2.1 Mycorrhizae in the Soil Community

A variety of elegant studies of soil enzymes and respiratory activity in relationship to soil physical and chemical properties and biological interactions (both belowground and aboveground) have been conducted. Some of this research has been directed at quantifying fungal and bacterial contributions to these activities through incorporation of population specific antibiotics (e.g. cycloheximide to inhibit fungal activity and streptomycin to control bacterial activity) into the laboratory-incubated soil–plant samples but evaluation of the role of mycorrhizal fungi in these soil functions is rarely considered. Standard methods for soil collection rarely preserve the soil–root–fungal associations. Hence, even direct counts of fungal mycelia in the soil would rarely, if ever, be linked to plant roots. This omission of a consideration of these important trophic interactions occurs in spite

of the knowledge that mycorrhizal fungi constitute a major portion of soil biomass and that these fungi extend throughout the soil profile, far beyond the regions classified as rhizosphere soil. An example of the association of soil enzymatic activity with root and fungal development was provided by Spalding et al. (1975). In their study, cellulase, invertase, polygalacturonase, and peroxidase activities were found to increase in soil under lycopodium fairy rings, suggesting a mycorrhizal fungal association with organic matter metabolism.

The potential for mycorrhizal contribution to total soil metabolic activities can be appreciated by examining examples of the quantities of biomass contained in these structures. Fogel and Hunt (1983), in a study of a young, second-growth Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) stand, found that mycorrhizae constituted 6% of the total tree standing crop. Furthermore, they found that roots and mycorrhizae contained larger reserves of nitrogen, phosphorus, potassium, and magnesium than did the forest floor or soil fungi. Fine roots and mycorrhizae contributed between 84% and 78% of the total tree organic matter to the soil. This study suggests that although the mycorrhizal fungal biomass only constitutes a small portion of the standing crop, it may account for 50% of the organic matter throughput in the *P. menziesii* stand. Read (1984) suggests that mycorrhizal fungal biomass may be the largest microbial biomass component of many forest soils. Allen (1991) states that "mycorrhizal fungi may be the single largest consumer group of net primary production in many, if not most, terrestrial biomes." Vesicular-arbuscular fungal hyphae have been quantified at densities of up to $38\,m\,cm^{-3}$ (Allen and Allen 1986). It must be remembered that although a portion of the fungal biomass is intimately associated with the root, the fungal net extends throughout the soil surface. (See Allen 1991 for a review of this topic.)

From these observations, a number of benefits of mycorrhizal development to the total soil microbial community can be delineated. Foremost among the contributions to the soil community is the capacity to cycle nutrients, i.e. mineralize accumulated biomass. Although most research efforts have been directed at quantifying mineral nutrient transfer from fungal biomass or soil to plant tissue, it is reasonable to assume that a portion of the metabolic products of this fungal activity will be released to the soil microbial community. For example, mycorrhizal fungi have been shown to mineralize soil organic phosphate through synthesis of phosphatase (e.g. Dighton 1983 and Pasqualini et al. 1992) and to solubolize mineral phosphorus through the acidification of the soil habitat through carbon dioxide production (see Knight et al. 1989).

Indirect benefits to the soil community by mycorrhizal fungal growth result from a generalized improvement of soil structure. Fungal hyphal development increases the association of soil particulates into aggregates (see Tisdall and Oades 1982 and for a review of this topic, Tate 1987). This improved soil structure has a direct impact on the indigenous microbial community through improved aeration

and moisture infiltration and an indirect effect via stimulation of plant root growth. Clearly, any augmentation of root development increases the quantities of fixed carbon reaching the soil microbial community.

8.2.2 Symbiont Benefits from Mycorrhizal Development

Mycorrhizal fungi are clearly instrumental in augmenting plant nutrient availability in nutrient-stressed ecosystems. A wide range of data (at times contradictory data) has been collected demonstrating nutritional benefits to plant communities in stressed communities through mycorrhizal symbiotic associations. Most research has shown improved nutrient transfer to the plant tissue through augmentation of the adsorbing surface of roots by extension of the fungal mycelium into nonrhizosphere soil. Gains in phosphate, nitrogen, and water transfers are most commonly reported. The expansive literature documenting these benefits has been reviewed by Allen (1991), Bagyarak (1991), Gupta (1991), and Mukerjii et al. (1991).

Perhaps in a more intriguing vein, mycorrhizal fungi may not only enhance soil–plant transfer of nutrients, but may also be instrumental in movement of nutrients between plants (e.g. Camel et al. 1991, Eason et al. 1991, Hamel et al. 1991, Read et al. 1989). Read et al. (1989) demonstrated through the use of $^{14}CO_2$ that carbon moves freely between plants connected by mycorrhizal mycelium. This plant–plant bridging by fungal hyphae occurs between host plants of the same or differing species. Similarly, Chiariello et al. (1982) found that ^{32}P-phosphate sprayed on leaves of *Plantago erecta* in grassland was transferred to the shoots of about 20% of close neighbors. Vesicular-arbuscular mycorrhizae were noted to connect the root systems of neighbors of different species and considered to have mediated the nutrient transfer. Camel et al. (1991) found that the hyphal front may advance at a rate of 2.3 cm week^{-1} in soil sand mixtures and that bridges of at least 90 mm may be formed. These workers suggest a competitive advantage of this bridging in plant groupings where mycorrhizal inoculum is limited. Plants developing in a less desirable portion of the ecosystem (e.g. a shaded site) may gain benefits of the photosynthetic activity of the less stressed members of the community as well as gains from nutrient production of the mycorrhizal function through an interconnected root system.

A further stimulus to plant biomass productivity is associated with the capacity of mycorrhizal associations to reduce or prevent plant disease development (see Jalali and Jalili 1991 for a review of this topic). The association of the fungus with plant disease-susceptible root tissue may reduce pathogen vulnerability through modification of exudate composition or concentration, stimulation of plant disease-protective response through its own infection of the root tissue, inhibit competing microbial populations directly through synthesis of antibiotics, and limit access of the plant pathogen to root tissue by physically occupying the root surface.

Enhancement of fungal biomass by the symbiotic association with root tissue is generally attributed to augmented availability of photosynthate in the form of simple sugars for fungal energy and carbon needs and reduced competition with the general rhizosphere microflora. Kucey and Paul (1982) found that within mycorrhizal, nodulated faba beans (*Vicia faba* L.) about 4% of the carbon fixed by the host was transferred to the fungal associate. (For a more detailed examination of this topic, see Allen 1991.) A competitive advantage for the fungal associate in dealing with soil microbial populations in general and more specifically rhizosphere microbes is derived from its close physical association with the host root. Intrusion into the interlameller space between cortical cells (as well as cell penetration by the endomycorrhizae) and intimate contact with the root surface facilitate nutrient recovery from the host tissue.

Of interest from the viewpoint of impact of the host plant on the behavior of mycorrhizal fungi in soil is the potential for selective stimulation of spore outgrowth. Published results regarding selective stimulation of fungal spore outgrowth by plant root products are mixed yet a real microsite stimulation most probably does occur. Bechard and Piche (1989) found that in their study of carrots and the vesicular-arbuscular mycorrhizal fungus *Gigaspora margarita*, root volatiles provided little stimulation of spore germination and that the exudates alone had no effect. Considerable stimulation was detected from the synergistic interaction between the volatile and exudate root products. From their study of the effect of root exudates of *Trifolium repans* on *Glomus fasiculatus* germination, Elias and Safir (1987) conclude that the primary stimulation of spore outgrowth results from exudates from phosphorus-deficient clover seedlings when compared to the effect of exudates from phosphorus-sufficient seedlings, suggesting that it is the quality of the exudates which affect the spore germination rates. Further studies (Nair et al. 1991) suggest that two isoflavonoids (7-hydroxy-4'-methoxy isoflavone and 5,7,-dihydroxy,4'-methoxy isoflavone) may be signal molecules in vesicular-arbuscular mycorrhizal symbioses. Other workers (see Bowen 1969) conclude that root exudates had no effect on spore outgrowth. It may be deduced from evaluation of these disparate observations that mycorrhizal fungal spores are sensitive to root exudates and that variability in composition (quality) of these plant products with host nutritional status greatly affects this interaction.

A further observation of the infection process in mycorrhizal development is a potential interaction of plant growth-promoting bacteria (e.g. Bowen and Theodorou 1979; Garbaue and Bowen 1989; Meyer and Linderman 1986a,b). Bowen and Theodorou (1979) found that a variety of bacteria could depress, have no effect on, or stimulate mycorrhizal development in the rhizoplane of *Pinus radiata*, suggesting the necessity of fungal compatibility with the rhizosphere bacterial populations. Garbaue and Bowen (1989) actually found that "helper" microorganisms exist in the rhizosphere that improved the efficiency of ectomycorrhizal infection of *Pi. radiata*

D. Don growing in a sandy podzol. Meyer and Linderman (1986a) found that a combination of plant growth-stimulating bacteria and mycorrhizal development of subterranean clover (*Trifolium subterraneum* L.) resulted in considerably greater plant biomass synthesis than with either microbial component taken singly.

8.2.3 Environmental Considerations

The statement by Allen (1991) that "Mycorrhizae represent one of the least understood, most widespread, and most important biological symbioses on Earth" is no more greatly appreciated than when application of this fungal–plant interaction to ecosystem problems is considered. Enlightenment about successful exploitation of mycorrhizal associations is gained by a consideration of the current applications of mycorrhizal interaction management (development of functional plant communities on disturbed sites – land reclamation, and inoculation of nursery stock for field planting) and an interesting but as yet little explored option (development of genetically engineered fungal associates for protection of host plants from chemical contaminants in their soil).

Although commonly considered from the viewpoint of industrially polluted soil, reclamation management to improve soil quality is more frequently related to "tired" agricultural soil systems (i.e. those cultivated in some cases for several centuries by methods designed to maximize crop yields at times at the sacrifice of maintaining soil quality). Traditionally, this soil management includes procedures which encourage a soil structure conducive to aboveground plant community development (see Tate 1987 for a review of this topic) and establishment of stable populations of organisms involved in biogeochemical cycling (see Tate 1985). Due to their ability to improve longevity and productivity of aboveground plant communities, mycorrhizal associations are a critical component in soil reclamation management.

Plant community gains from management of mycorrhizal associations for soil quality improvement are derived from both soil structural enhancements resulting from fungal contributions to soil aggregate formation and the improved availability of essential plant nutrients. Rapid and somewhat long-lasting benefits accrue from amendment of degraded soil with a variety of organic matter sources and fertilizers (e.g. Fresquez et al. 1990; Stroo and Jencks 1985; Tester 1990; Wong and Ho 1991), but for minimization of anthropogenic intervention, a stable soil microbe–plant interactive community must be developed. As indicated above, mycorrhizal associations are best developed under stressed conditions, especially phosphate stress. Examples of additional limitations common to degraded soils which encourage mycorrhizal symbioses development include fertilizer application practice and cropping system (Ellis et al. 1992), soil erosion (i.e. loss of fungal propagules with surface soil losses; Day et al. 1987), vegetation (Newman et al. 1981), and soil organic matter (Harvey et al. 1981). Hence, for rapid, and enduring

development of a reclaimed ecosystem, site management should include inclusion of fungal propagules in the reclamation target soil, implementation of plant nutrient management procedures which will not prevent mycorrhizal development, and where possible, utilization of inoculated transplants for establishment of aboveground plant communities.

Questions associated with inculation of seedlings with mycorrhizal fungal propagules relate not to the benefit of such associations to plant survival and productivity – these have long been assumed to be major pluses – but rather to optimization of inoculation processes and selection of the best fungal strains for the symbioses. For example, Marx et al. (1978) documented stimulation of loblolly pine seedlings by a variety of ectomycorrhizal fungi whereas more recently Theodorou and Bowen (1987) evaluated the selectivity of basidiospore germination and selection by pine seedlings and development of an effective method for inoculum conservation in the absence of host plants. Duponnois and Garbaye (1991) investigated the utilization of mycorrhization helper bacteria in establishing ectomycorrhizal fungal associations in forest nurseries. The unquestionable utility of such inoculation is exemplified by studies of Lindemann et al. (1984), where mycorrhizal interactions among other soil microbial activities were evaluated in mine spoil reclamation, and of Wilson et al. (1991) where mycorrhizae were utilized to reclaim portions of a dewatered flue-gas desulfurization sludge pond.

Future considerations of the utility of exploiting mycorrhizal associations for soil reclamation may also be based on extension of genetic engineering procedures to mycorrhizal fungi and modification of the carbon substrate metabolism capacity of these organisms. As was discussed above, mycorrhizal fungi extend from the growing root into the surrounding soil. The soil particle-associated mycorrhizal fungal hyphae catabolize soil organic carbon components. Clearly, potential exists to develop mycorrhizal fungi with the capacity to decompose pollutants that may occur in the vicinity of the roots of plants selected for soil reclamation (e.g. pine trees and associated shrubs) or even crop plants. The objective for the first situation would be to develop fungal associates that provide a benefit to the plant beyond those generally associated with mycorrhizal interactions, i.e. removal of potential plant toxicants. In the case of the crop plant, the fungi could serve as a "first line of defense" *per se*, thereby preventing toxicants which may contaminate food plants from encountering and being accumulated in sensitive root tissue.

8.3 The Mycorrhizosphere

Traditionally, rhizosphere activity and mycorrhizal symbioses have been studied as if they represented separate ecosystem components but the preceding discussion of these ecosystem phenomena leads at least to the suspicion that they occur

concurrently on many plant roots rather than as distinct entities. In all soil systems, a growing plant will develop some sort of microbial–plant interaction termed a rhizosphere. As our understanding of mycorrhizal symbioses expands, it is becoming more obvious that the vast majority of higher plants are mycorrhizal. Thus, we cannot conclude that all rhizospheres are mycorrhizospheres, but it is also evident that most of the data collected from field studies of rhizosphere populations must have resulted from evaluation of mycorrhizospheres – with the experimenter being ignorant of the manifestation of fungal mycelium in association with the roots, or not possessing the requisite training to differentiate mycorrhizal root structures. This conceptual oversight is gradually being corrected.

In evaluating the quantities of exudates and their chemical substituents, quantification of the intensity of mycorrhizal association is necessary. With symbiotic fungal–plant associations, essentially the entire root surface may be covered by several layers of fungal mycelium (ectomycorrhizal associations) or a major portion of the root surface may be free of obvious fungal hyphal development with the fungus deriving its nutrient through intracellular penetration (endomycorrhizal associations). In either case, the heterotrophic fungi consume root exudates plus the organic carbon contained in sloughed cells, thereby altering both the quality and quantity of root exudates. Some components of the exudate may be decreased or totally depleted.

A further complexity of carbon cycling in the more commonly occurring root soil complex, the mycorrhizosphere, is that one reservoir of fixed carbon in the soil is overlooked. Plant carbon is converted into the mycorrhizal fungal biomass. This carbon pool extends far beyond the rhizosphere, thus serving as a vehicle to translocate carbon from the plant to the soil community in general, following death of the fungal symbiont (see Fogel 1988 for further discussion of this topic). Therefore, it must be observed that the total nature of the microbial populations in the region of the root is altered by the presence of the fungal symbiont. Direct and indirect impacts of this interaction between the fungus and root exudates on the rhizosphere and rhizoplane microbial populations include determination of the active species and alteration of the total microbial biomass produced (e.g. Meyer and Linderman 1986b), as well as the control of plant pathogen interactions. These microbial consequences of fungal symbiont and rhizosphere bacteria interactions reflect back to the plant in that they may result in improved plant growth (Azcon 1989; Will and Silvia 1990)

These limited evaluations of the impact of both rhizosphere populations and mycorrhizal associations with plant growth emphasize the need to expand future root community studies to include all levels of trophic interactions – be they the result of symbiotic fungi, nitrogen fixation symbioses (*Rhizobium*–legume or actinorrhizal) or general rhizosphere bacterial, fungal, protozoan, or nematode population growth. Each of these entities withdraws nutrients from the common

source – plant photosynthate – either directly or indirectly, produce biomass, and alter the productivity or lack thereof of their neighbors. Ultimately, total ecosystem dynamics are controlled by the complex interactions of each member of the soil community.

8.4 Conclusion

The rhizosphere is most certainly an oasis of biological activity within the soil ecosystem. This ecosystem is represented by a diversity of microbial populations, the complete range of plant–microbe and microbe–microbe interactions, and the relative inclusiveness of all essential biogeochemical processes for total ecosystem development. An overall view of the processes occurring in the rhizosphere/mycorrhizosphere may be summarized by three words – interactions, productivity, and protection. That is, the rhizosphere is populated by a vast variety of life forms each of which is impacted both positively and negatively by all other creatures existing in the system; the productivity of the microbial community as well as the aboveground community is interlocked with the viability and stability of the microbial community (i.e. neither the plant community or the microbial components can function optimally singly); and lastly, the microbial community confers a resistance to the plant component of the system by competing with and in many cases destroying potentially harmful plant pathogens.

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9

Introduction to the Biogeochemical Cycles

Consider a handful of soil. Most soils (i.e. mineral soils) generally have the look and feel of a complex mixture of mineral particles; that is, they are composed of a reasonably simple mixture of sand, silt, and clay. This observation could easily lead to the conclusion that soil is simply a mixture of mineral particulates. Perhaps we might also conclude that variability in soil function could be derived, at least in part, from differences in chemical composition and particulate size. A bit more contemplation could lead to the conclusion that the role of soil in the ecosystem only extends to the properties associated with providing physical support for plants and directing water and air to plant roots. Over the millennia, we have come to appreciate the fact that the easily seen soil components are closely associated with myriad organic compounds and living organisms ranging from nano-sized bacteria and viruses to easily recognized higher animal and plant life. Complex biogeochemical cycles of life requiring minerals and mineralization of decaying biomass provide the energy and nutrients required for growth of the soil biological community. Thus, this chapter has the objectives of examining the relationships held in common by organisms producing the nutrients and the processes used in their study.

As our examination of soil properties extends beyond our initial observations of the complexity of the physical structure, we must ask the simple question "Why is such complexity in structure necessary?" The answer to this query relies on considerations beyond examination of individual soil minerals. The role of soil in relationship to the function (existence) of the total ecosystem must be considered. A good starting point could be the contributions of soil to plant community growth and development. Clearly, soil must provide a physical support for plants as well as containing the basic elements required for plant growth. Some initial conclusions regarding soil structure and variability include the following.

- The nutrient needs of the plants change throughout the growing season.
- Temperature in the soil change daily.

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- Soil moisture is highly variable.
- Growth of the plant changes soil chemical and physical properties.

More succinctly stated, variability in soil chemical, biological, and physical properties necessitates changes in soil properties required to maintain a vibrant, productive plant community just as the aboveground plant community must evolve to sustain its development and resilience.

Therefore, it must be concluded that the complexity of soil components and their interactions supports (i) the resistance of the ecosystem to external perturbations, (ii) the productivity of the currently existing living community, and (iii) the sustainability of the ecosystem. That is, the answer to our simple question regarding the necessity of such a complex mixture of soil particles and life could be reduced to the simple statement "no complex, evolving soil system – no terrestrial life." This conclusion is further supported by a short list of soil services involving soil microbes, examples of the function of soil organic matter (humus), and the roles of soil aggregates as an introduction to the importance of the interaction of soil physical, chemical, and biological processes.

An important yet perhaps not obvious conclusion revealed by this introduction is that a primary driving force for the cycling of nutrients in soil is the capacity of the soil biological community to mineralize plant organic matter for the energy and nutrients required for replication. Further analysis will show that transformations supportive of the biogeochemical cycles involve external organic matter sources (plant biomass) as well as that produced through chemical and biological reactions within the soil itself.

Thus, basic questions arising at this point include "What is soil organic matter (humus) and how does it differ from the plant biomass, the primary source of nutrients and energy supporting growth of the soil biological community?" The major groupings of organic matter makingg up humus include (i) partially decomposed plant debris, (ii) microbial biomass, (iii) identifiable classes of biochemicals (e.g. cellulose, lignins, etc.) that have been separated from the cells synthesizing them, and (iv) humic substances. The complexity of organic chemicals derived from plant and animal biomass is reasonably familiar; humic substances may be somewhat less familiar. This rather cursory examination of common soil organic components leads to the conclusion that they are derived from transformations of native plant and animal biomass, humic substances formed *in situ*, human waste materials, and anthropogenic chemicals.

At this point of our analysis, we can conclude that the diversity of organic chemicals and their fate in the soil system presents a potentially overwhelming task for those charged with examining nutrient cycling in soils. Thus the primary objective of this chapter is to provide a basic understanding of biogeochemical cycles through evaluating the general properties of the cycles and examining the

interrelationships of these processes with the goal of developing an appreciation of the importance of these processes to overall ecosystem function. Specific biogeochemical cycles are examined in greater detail in subsequent chapters.

9.1 Introduction to Conceptual and Mathematical Models of Biogeochemical Cycles

A primary maxim for this analysis of biogeochemical cycles is that the energy and nutrients used by soil microbial biomass are derived from microbial recovery of the nutrients and energy contained in aboveground plant biomass. That is, one could state that since the majority of the soil microbial biomass results from mineralization of photosynthetically produced plant biomass, the soil biological community is ultimately solar powered, with the primary pathway for energy inputs to support belowground life being solar-powered plant biomass synthesis to soil primary producers (e.g. bacteria and fungi). Other natural sources of microbial nutrients and energy include animal biomass as well as nutrients contained in rainwater and other surface water, although to a much lower degree than the plant biomass. In systems with high levels of human intrusion, products of societal development and function also play a meaningful role in this nutrient cycling and energy transformation.

A more complete consideration of the organic substances supporting soil systems therefore requires an understanding of transformations of the biochemical components of living biomass and anthropogenically synthesized organics, as well as the more biodegradation-resistant substances such as humic substances as well as lignin. Elucidation of the processes associated with the building blocks of microbial biomass requires study of the chemical nature, physical location within the soil system, and its degradation susceptibility. For plant biomass, some components are easily converted into microbial biomass and energy (e.g. carbohydrates and proteins), whereas others are not (e.g. lignin). Furthermore, meaningful quantities of the organic compounds entering soil are transformed biologically as well as chemically into complex organic materials (e.g. humic acid, fulvic acids, and humin) that are not easily metabolized by the soil microbial community. Understanding the nature of the transformation of soil and plant organic matter as well as a wide variety of human-synthesized organic components amended to soil requires a basic understanding of the variable physical states of these materials within soil and the extremely variable distributions of organic substances within the soil matrix. For example, as noted in earlier chapters, organic matter may be occluded to the microbial community by its incorporation into soil aggregates, binding to clay, or being contained within resting microbial cells.

9.1.1 Development and Utility of Conceptual Models

Elucidation of the diversity of organic substances and their interactions with the soil physical structure is essential for developing appropriate soil management plans, determining sustainability of the soil system, and evaluating the impact of transformation of organic matter on external processes, such as mediating climate change. Among the tools commonly used for understanding the distribution of organic and inorganic nutrients within the complex soil matrix and their role in soil, as well as in total ecosystem stability and function, are a variety of conceptual and mathematical models.

Conceptual models of biogeochemical cycles are valuable for evaluating and testing our understanding of nutrient movement and distribution in an ecosystem. The degree of complexity of individual models necessarily varies with the objectives of the model developer. Rarely are all states (chemical as well as physical) of the nutrient pools represented in a specific model. Rather, those processes most important to understanding a particular ecosystem function are detailed. For example, a model of carbon cycling can be as simple as a depiction of the size of the carbon dioxide and organic carbon pools and assessment of the rates of movement of carbon between the two pools. Alternatively, movement of carbon between mineral (e.g. carbon dioxide and limestone) and soil organic matter, microbial biomass, and plant biomass pools may be evaluated in order to gain a better picture of the fate of soil organic components entering the soil system. Should specific organic compounds be of interest, the model may be concentrated on distributions and fate of specific chemical compounds (e.g. carbon dioxide, cellulose, lignin, and proteins).

The variability in the degree of complexity associated with development of biogeochemical cycles occurring in soil reflects the vast diversity of chemical and physical states of the elements (e.g. carbon, nitrogen, sulfur, phosphorus) that can be considered. Carbon is found as carbon dioxide in soil air, bicarbonate in soil water, and carbonate in soil minerals, not to mention the vast number of organic chemicals contained in living cells, soil humus, and any anthropogenic or xenobiotic compounds in the system. Added complexity is introduced when the elements of concerns exists in several oxidation states (e. g., nitrogen, sulfur as well as metal ions such as iron, and aluminum. Therefore, an evaluation of biogeochemical cycles must include consideration of the totality of biological, chemical, and physical interactions associated with the movement of nutrients between organic and inorganic pools and the variety of geochemical reservoirs existent within an ecosystem.

This definition of the complete realm of biogeochemical cycles emphasizes the point that study of these chemical transformations has real implications beyond the world of plant science and general biology. Study of these cycles of life-sustaining nutrients is also essential for such broad-based disciplines as general ecology or

environmental science. For example, consideration of biogeochemical cycles in disturbed environments is essential for optimizing anthropogenic activities for ecosystem sustenance as well as in development of management plans for restoration or reclamation of damaged systems; that is, development of appropriate ecosystem stewardship procedures.

As suggested by the foregoing analysis, a study of biogeochemical cycles requires a means of assessing a wide array of complex interactions within a constantly varying environment. Some details may be studied in isolation, but it must be remembered that the primary objective of the exercise is to develop an understanding of the overall pattern of distribution and movement of nutrients in the ecosystem and their impact on ecosystem function and sustainability as well as evolution. A means of developing an overarching framework for study of biogeochemical cycles is to assemble the data into models. These models may be used to elucidate gaps in knowledge, provide hypotheses for future study, and develop experimental plans for quantifying nutrient movement within the cycles and assessment of the impact of environmental modification on these processes. Early modeling activity, or perhaps modeling activity at its simplest, involves conceptual models. The substances of interest are grouped in boxes (commonly referred to as pools) with arrows between the boxes to designate direction of nutrient flow. Some models include size and shape of the vectors of movement of the substances of interest between various chemical and physical pools existent in the site of interest. These vectors may involve processes of limited magnitude (e.g. decomposition of specific pesticides) as represented by major or minor pathways of nutrient flow (or in the example noted above, movement of the pesticide between various physical and chemical states of the chemical and the resultant changes in its impact of ecosystem function). These models provide a basis for design of experiments that yield the data necessary for development of more complex and predictive mathematical models.

Therefore, common objectives of study of nutrient flow in an ecosystem may include (i) the assessment of chemical forms of a nutrient element (e.g. carbon as carbon dioxide and organic matter; nitrogen as mineral and organic forms; phosphorus in soil minerals, water-soluble, and organic forms) in an ecosystem of interest, (ii) the quantity of nutrients retained in these compounds (i.e. the size of the nutrient pool), and (iii) the rate of movement of the nutrients between these various nutrient pools as they affect the function and stability of various biological populations.

Once the participants of interest in biogeochemical cycles are determined (even in lieu of determination of all the pertinent chemical intermediates), conceptual models may be assembled, which are most easily presented as a series of boxes representative of the primary nutrient pools with arrows connecting the boxes to indicate the directional transformation (i.e. nutrient flux) of the cycle components (see Figure 9.1 for a depiction of the carbon cycle). To augment the information contained in a conceptual model, the type of arrow connecting the various

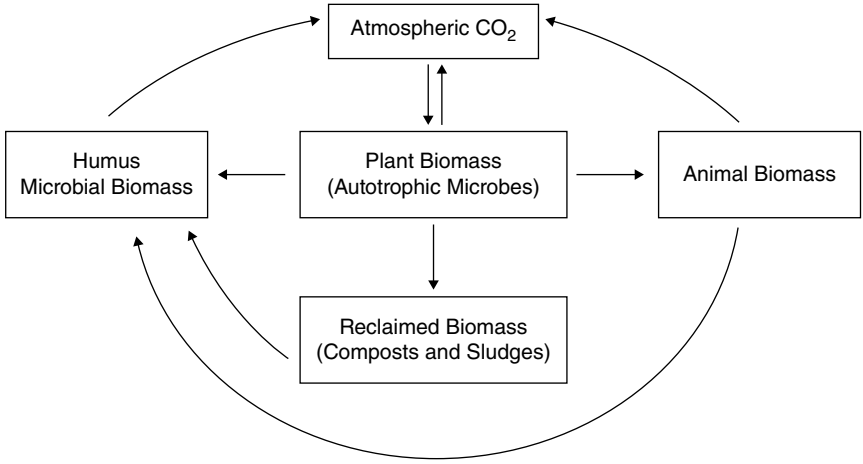

Figure 9.1 A generalized carbon cycle model with biological and atmospheric aspects emphasized.

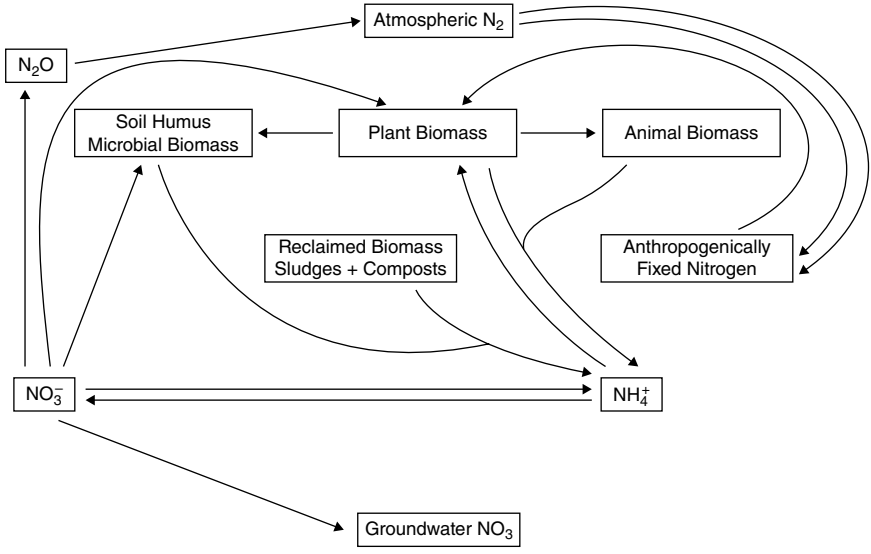

Figure 9.2 A generalized model of the nitrogen cycle.

reservoirs (e.g. dotted, size differential, or boldface) may be varied to suggest differences in the magnitude of the processes depicted. More commonly, a conceptual model is limited to indication of the nutrient reservoir and the direction of nutrient flow. Details such as distinction between relatively stable reservoirs, such

as soil minerals or peat deposits, and the more ephemeral cycle components exemplified by simple organic compounds or mineral nitrogen forms may be minimal. That is, organic carbon may be considered as a single pool in the cycle or perhaps two pools, representing slowly and rapidly metabolized carbon compounds. A more quantitative representation of biogeochemical cycles or the individual reactions comprising them is entailed in extension of conceptual models into mathematical models as described in Section 9.1.2.

Conceptual models of biogeochemical cycles emphasize the distribution of an element in a variety of chemical forms. The elements of interest are found to move between several chemical (organic vs inorganic) and/or oxidation states at predictable rates. Basic chemical components of plant and animal biomass are transformed from biomass unavailable forms to compounds that are readily incorporated into living cells. For example, the reasonably inert compound dinitrogen (N_2) is reduced by soil microbes to ammonium, which, once it is released by the microbial cell, can be incorporated into plant tissue. With the death of the plant, the soil microbial community may return the nitrogen to the atmospheric pool of dinitrogen. This conceptualization of biogeochemical cycles is plant centered (or perhaps life centered, when microbial and animal biomass are also viewed as major nutrient recipients). Other models could be centered on movement of elements and compounds to and from atmospheric pools, soil minerals, humus, or any other primary ecosystem component, such as production and decomposition of greenhouse gases.

Conceptual models by their very design may provide a biased view of biogeochemical cycles in that most representations are limited to designation of the basic chemical and biological forms of the cycle components. To provide a more complete model for soil microbiological or environmental science considerations, designation of the path for movement of the elements of interest through various ecosystem components (i.e. soil minerals, atmospheric gases, and biotic entities) is useful. A sound ecological approach to study of biogeochemical cycles should include an understanding of:

- the interactions between the compounds transformed, the biological community catalyzing the processes, and the physical environment in which the reactions occur and which itself may be modified during the process
- the contribution of spontaneous chemical reactions to the overall nutrient generation rates.

In regard to specific transformations, the primary product of the physical, chemical, and biological processes comprising a biogeochemical cycle is a stable, sustainable ecosystem (that is, an ecosystem that can adapt to changes in the physical, chemical, and biological controllers of the soil biological community). Thus, although conceptual models of biogeochemical cycles are usually presented as "boxes connected by arrows," it must be remembered that the boxes represent

"real-world" nutrient reservoirs, such as plant and microbial biomass. Furthermore, at least as important as the quantification of chemical form in a cycle is an understanding of the magnitude of the vectors representing the movement of the material of interest between the various nutrient pools. The importance of a large pool of nutrients (such as atmospheric N_2), which in itself is unavailable to microbes, is determined not by its size but rather by the rate at which it is converted into a form useable by microbes (i.e. nitrogen fixation). The rate of the transformation is controlled not only by biological processes themselves but also by the properties of the environment in which the plant, microbe, or animal is located. Therefore, the occurrence of biogeochemical cycles is imperative for long-term sustainability of the ecosystem.

Thus, collection of data for development of conceptual models should entail consideration of biotic as well as abiotic interactions. For example, mineral nitrogen (i.e. ammonium and nitrate) availability frequently controls the productivity and nature of the aboveground community. This nitrogen may be derived through nitrogen fixation or other sources external to the specific ecosystem under study or the nitrogen contained in biomass may be recycled into nascent biomass through mineralization of deceased biomass and soil organic matter pools. Thus, in this situation, the indigenous nitrogen mineralization rate is a primary controlling factor for biomass production. Note that in the latter situation, the total amount of nitrogen contained within the soil system may be reasonably constant. That is, the productivity of the aboveground community is limited at least in part by the capacity of the soil microbial community (nitrogen fixers or nitrogen mineralizers) to incorporate the soil nitrate and ammonium into plant biomass. The activity of this soil microbial community is in turn controlled by the quantities of energy and nutrients provided by the aboveground community in the form of photosynthate, as well as that contained in the soil physical and chemical environment. For example, in a high-moisture, high-clay soil, limitation of plant productivity by the physical properties of the system can also curtail soil microbial activity, which is dependent on decomposition of plant biomass for its energy source. Also, anoxic conditions may favor loss of fixed nitrogen from the system through biological denitrification reactions.

These life-limiting environmental properties are continually being modified by the physical presence and biological products of the plant, animal, and microbial communities (see Tate 1987). For example, improvements in the soil physical environment through enhancement of soil aggregate structure by growth of the microbial community augments plant biomass production, which may further stimulate microbial growth through augmented inputs of fixed carbon.

Therefore, essential steps in the development and refinement of conceptual models involve elucidation of the forms of the nutrient element of interest in the system, perhaps tracing the movement of elements between these forms using such isotopes as ^{14}C or ^{15}N, and determination of the physical and chemical properties of the system controlling the relative magnitude of the various chemical pools

and the rate of flux between these reservoirs (e.g. see Paterson et al. 2011; Tatzber et al. 2009) The process of model building is based primarily on characterization of the system and development of a reasonable understanding of the relationship between the various nutrient reservoirs occurring therein. These studies may entail determination of biological trophic interactions (e.g. Pennisi 2015; Santos et al. 1981, 1984; Santos and Whitford 1981), determination of the impact of management and climate change on ecosystem function (e.g. Allen 1990; Pastor and Post 1988; Tabatabai and Chae 1991; Walters et al. 1992), evaluation of the impact of properties of ecosystem inputs on their mineralization (e.g. Fresquez et al. 1990; Meentemeyer 1978), as well as examination of native ecosystem processes (e.g. Parker et al. 1983).

9.1.2 Mathematical Modeling of Biogeochemical Cycles

Common objectives of projects examining nutrient cycling in a soil ecosystem are twofold.

- Developing an understanding of the processes and their limitations in the particular site of interest. This type of experiment may involve achieving a clearer understanding of the nuances of nutrient availability in a native forest ecosystem, for example. Alternatively, these types of studies can provide the basis for nutrient management as exemplified in agricultural soils as well as for soil reclamation.
- Collection of a data pool that allows extension of the concepts learned at a few sites to a more extensive land area.

Conceptual and mathematical models aid in achievement of these types of objectives. A conceptual model provides a basis for the "design" of the mathematical representation of the biogeochemical cycles in an ecosystem. The components of the cycle and direction of flow of nutrients are described, but little is revealed about the size of the various pools or the magnitude of flow between the pools. Frequently, a conceptual model can represent a hypothesis of "how the system works," thereby providing a basis for an experiment designed to test specific hypotheses. Initial steps for attaining an understanding of nutrient cycling processes include analysis of the quantities of various compounds present within the soil site of interest. Generally, it becomes clear that a detailed understanding of the intricacies of the reactions is obscured by site heterogeneity and the sheer number of potential soil properties that control the reaction rates and nutrient distribution. Thus, a mathematical approach to data summarization and interpretation is required.

First steps for developing more generally applicable mathematical relationships may stem from application of statistical analysis procedures. A simple model may be yielded through a linear or nonlinear regression analysis of the data. The resultant equations may be reasonably descriptive of the particular site under

study, but applicability to other situations is limited. This conclusion stems from the fact that the equations derived are mathematical relationships fitted to aspecific data sets. That is, the relationship may be forced to fit a straight line, parabola, or hyperbola with the constants in the equations being representative of the conditions of the limited number of soil sites used in the experiment.

Equations more generally applied to biological, chemical, or physical reactions may be adapted to modeling environmental data. For example, an enzyme-catalyzed reaction may be reasonably easily described by a first-order equation or in some cases by a Michaelis–Menten relationship (see Chapter 6). As the complexity of the system increases (e.g. interest in the quantities of the reactant consumed within the profile of a conifer forest) and the variety of interactions with soil properties and climatic variations expands, inclusion of more general relationships, such as the Q_{10} equation or Ahrenius relationship for temperature impact on reaction rates, may be necessary to increase model utility. Furthermore, in the complex, heterogeneous environment of soil, effective chemical concentrations for a reaction may be defined by properties other than total substrate present. For example, reaction rates may be dependent upon soil parameters such as the rate of dissolution of the substrate (Stucki and Alexander 1987) or diffusion of the enzyme substrate into the microsite wherein the organisms reside (Scow and Alexander 1992; Scow and Hutson 1992). Also, in an ecosystem adapting to a perturbation, the rate of occurrence of a process may be proportional to the growth or death of the microbial populations producing the requisite enzymes (i.e. the reaction may be best described by a Monod equation – see Chapter 4).

Therefore, a variety of mathematical relationships descriptive of not only biological but also chemical and physical interactions are combined to provide an interactive series of equations descriptive of the extent of a natural process and predictive of rate changes with perturbation of the system. Because of the general applicability of the mathematical relationships (e.g. Michaelis–Menton equations provide a reasonable model of the kinetics of the vast majority of enzymatic processes), biogeochemical models based on them provide reasonable descriptors of process rates in a diversity of situations. It must be remembered that applicability of the mathematical relationship to alternative situations is still limited by the range of data used to validate the model. For example, a model descriptive of nitrogen transformation rates in well-drained agricultural soils would have to be shown to be applicable to swamp or wetland soils were such soils of significant importances in the sites being studied. This situation results from the fact that the mathematical relationships used to describe soil processes are a mixture of variables and constants. Values for the variables are measured, estimated, or assumed from analysis of actual soil situations. The constants are based on or are commonly derived from these "real-world" studies. Thus, equations and models are only applicable within the range of variation of the data used to develop (validate) them. Their applicability to other soils with properties outside that range must be

proven. A simple mathematical model of soil nitrogen mineralization rates and organic nitrogen pool size, which is based on first-order reaction kinetics, is described in Section 9.5.4.

Examples of the diversity of opportunities wherein studies of soil microbial interactions with the physical and chemical structure of their habit can benefit from development of simple to complex conceptual and mathematical models include the following.

- Assessment and elucidation of soil properties controlling specific portions of a biogeochemical cycle. Examples of such applications include study of the impact of distribution of aerobic and anaerobic microsites on denitrification rates (McConnaughey and Bouldin 1985a, 1985b; McConnaughey et al. 1985); sequestration of the chemical(s) of interest, and thereby reduction of availability of decomposable organic matter to soil microbes (see Dou et al. 2014; Wang and Sainju 2014), as well as evaluation of nitrogen mineralization (Deans et al. 1986), sulfur and nitrogen mineralization (Ellert and Bettany 1988) and carbon mineralization (Stroo et al. 1989) in a variety of ecosystems.
- Evaluation of ecosystem response to global changes such as determination of soil changes due to global warming (see Creamer et al. 2015; Jenkinson et al. 1991; Lal 2004; Luo et al. 2014).
- Quantification of specific fluxes between nutrient pools, for example, evaluation of the importance of nutrients contained in microbial biomass in nutrient cycling (Trumbore and Czimezik 2008; van Veen et al. 1984).
- Prediction of the impact of management changes on soil enzyme activity, microbial populations, and soil quality-associated properties, such as nutrient levels and their distribution, variation in soil enzyme activities and microbial population distribution and function (Hunt 1977; Kennedy and Schillinger 2006; Parton et al. 1988; Paterson et al. 2011; Stott et al. 2013; Wallenius et al. 2011), or of erosion on plant phosphorus availability (Jones et al. 1984).

In each of these situations, varying combinations of mathematical relationships descriptive of biological transformations, pH variation, temperature effects on biological processes, adsorption processes, plant composition were combined to provide a mathematical representation of movement of nutrients between various biogeochemical nutrient pools.

9.2 Specific Models of Biogeochemical Cycles and Their Application

Basic conceptual models of the primary nutrient cycles (carbon, nitrogen, sulfur, and phosphorus) are depicted in Figures 9.1–9.4 respectively. These models are designed to emphasize the essential components of the cycles. More obvious

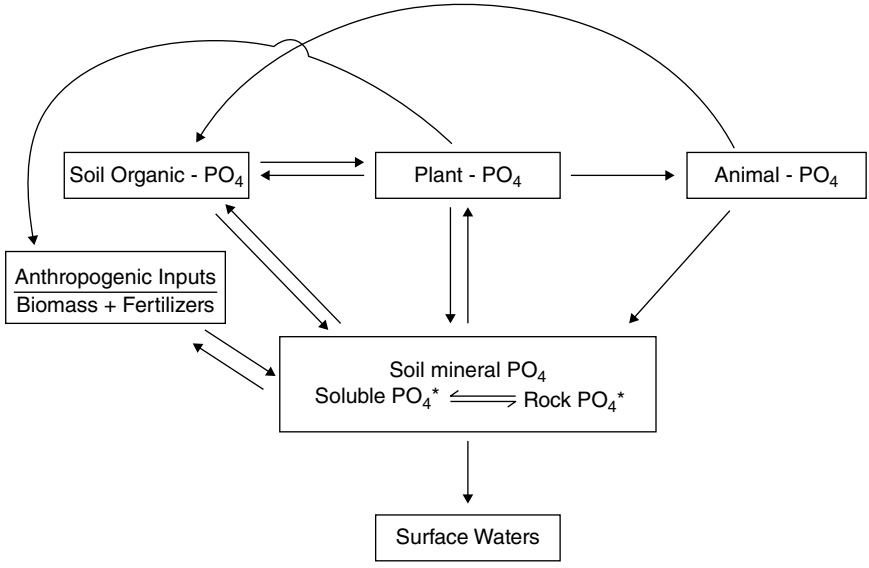

Figure 9.3 A model of the soil phosphorus cycle with soil mineral reservoirs as the central component.

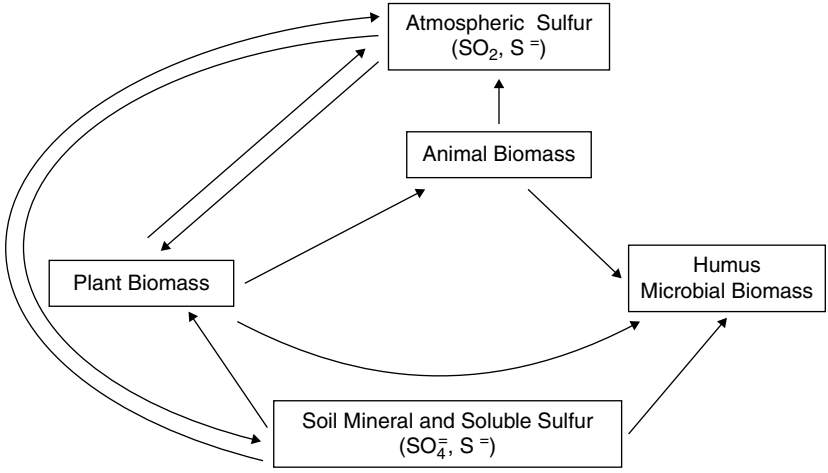

Figure 9.4 A model of the sulfur cycle showing generalized interactions of biological resources with soil and atmospheric sources.

points to note are that the elements are transferred between organic and inorganic forms (all cycles) as well as through a variety of oxidation states (nitrogen and sulfur cycles). The oxidation state of nitrogen varies from −3 (ammonium ion) to +5 (nitrate ion) whereas in the sulfur cycle the oxidation state of the sulfur atom

varies from −2 (sulfide) to +6 (sulfate). Similarly, a variety of soil and atmospheric inorganic compounds (e.g. nitrate, ammonium, dinitrogen, elemental sulfur, phosphate rock, sulfur-bearing minerals) and plant, animal, and microbial biomass organic components constitute primary nutrient pools in each of the models. Note that geological materials are of major importance in both the sulfur and phosphorus cycles.

Traditionally, biogeochemical cycles are studied in order to assess the relationship of nutrient state to higher plant productivity. This limited purview could lead to an underappreciation of the impact of nutrient cycling on total ecosystem functions and properties. A number of intermediates in the various cycles have considerable impact on the integrity of the terrestrial ecosystem. Positive as well as negative implications are discerned, especially from the viewpoint of societal impact on system function.

Perturbations of nutrient cycling could result in overproduction of substances that result in large-scale declines in system quality, altered biomass productivity, or modified terrestrial ecosystem sustainability. A long-appreciated example of this phenomenon is the changes in soil organic matter reserves due to soil cultivation (Tate 1987; Rosenzweiz and Hillel 1998) as well as total plant biomass productivity (e.g. Adams et al. 1990; Allen 1990; Dahlman et al. 1985; Reddy et al. 1989). Similarly, it has long been noted that soil organic nitrogen and carbon levels are generally enhanced when conservation tillage practices are implemented on soils previously managed with conventional tillage methods (e.g. Blevins et al. 1984; Cambardella and Elliott 1992; Dou et al. 2014; Doran 1980; Kennedy and Schillinger 2006). Loss of soil organic matter due to soil cultivation increases atmospheric carbon dioxide levels, thereby impacting global warming. In contrast, increased storage of carbon in soil organic matter pools moderates global warming due to greenhouse gas production. Furthermore, sulfate reduction to sulfide may be exploited to reduce the difficulties associated with acid mine drainage-impacted soils, and denitrification may be stimulated to reduce surface and groundwater contamination with nitrate.

The ultimate impact of the soil microbial community on the total ecosystem is derived from its contribution to the orderly transition of the chemicals of life through their various oxidation states. Optimization of ecosystem conditions for nutrient cycling results in development of a sustainable system in which nutrients are cycled with minimal loss of essential components from the system or movement of potentially troublesome intermediates into portions of the ecosystem where their presence may be less appropriate (e.g. resulting in lake eutrophication or groundwater contamination). Microorganisms are instrumental in such essential ecosystem-sustaining activities via the conversion of plant-unavailable forms (e.g. N_2) to chemical entities that may be incorporated into plant biomass (nitrate (NO_3^-) or ammonium (NH_4^+)) as well as the return of organic components to inorganic forms, which may be recycled into plant biomass (carbon or nitrogen

mineralization). Loss of plant biomass nutrients phosphate or nitrate negatively impacts the system by reducing its productivity and could be damaging to adjacent surface waters. Soil management to balance mineral nitrogen production and consumption minimizes surface- and groundwater quality decline.

9.2.1 The Environmental Connection

To truly understand the ramifications of any biogeochemical process, the implications of the synergism between the soil biota and their environment must be considered. Both the physical environment and the biological entities growing therein are modified by the products of biological growth. This synergism is absolutely essential for development of a sustainable ecosystem. In a managed site, such as a reclamation project, the sustainability of the developing ecosystem is reliant upon the ability of the microbial community to modify an initially hostile habitat to one more conducive for its own growth and sustainability as well as for the higher plant community. In highly damaged sites or in situations where rapid establishment of an esthetic aboveground community is desirable or required, anthropogenic intervention to manage microbially mediated processes may be necessary. In the absence of such site management, the longevity of the biological populations essential for establishment of a viable aboveground and belowground community could easily be truncated.

A typical scenario could be as follows. To provide a rapid start to the soil microbial community and to sustain aboveground populations until soil physical conditions are modified to those more conducive to plant development, the soils may be amended with an organic material (such as composts and sludges), nitrogenous fertilizers, and limestone to stimulate development of the indigenous microbial community. The organic amendments provide energy to the microbial communities as well as improving their growth conditions by complexing of heavy metals. The nitrogenous fertilizers are used to address imbalances in the carbon to nitrogen ratios of the organic components of the system. Liming of the soil may be necessary to adjust the soil pH to a range optimal for biological community development. Initially, this managed ecosystem development would be supported by the external nutrient inputs. A stable situation requires the slow evolution of soil populations and modification of soil physical properties to a point where nutrients required for continued biomass productions are produced internally. This transition from an artificially sustained to an internally sufficient system requires such physical-biological synergistic interactions as enhancement of soil physical structure through aggregation (see Chapter 1), as well as microbial amelioration of soil acidity in sulfate-impacted sites. This system development can be termed quasi-synergistic in that a feedback interaction occurs between the developing microbial community and its physical environment. As the complexity of the

microbial community increases, one product of its activity is an improvement in the physical and chemical conditions of its habitat. This alteration in turn supports further microbial growth. This cycle is repeated until ultimately a steady state develops with an optimized balance between the microbes and their physical and chemical environment.

These belowground processes not only optimize soil microbial community activity and development, but also improve conditions for plant population productivity and longevity. The development of soil aggregates through the action of soil microbes results in improved air diffusion and water infiltration rates. Therefore, not only is the soil microbial community stimulated by this habitat improvement but plant root growth and aboveground biomass synthesis are also increased. The response of the plant community to the microbially modified soil structure allows for further enhancement of soil microbial activity. The microbial community is limited, to a large degree, by the fixed carbon supply. This fixed carbon in nonmanaged ecosystems is the direct product of plant photosynthesis. Therefore, the improvement of soil structure noted above results in better conditions for plant growth, which further stimulates the microbial community through the enhanced input of photosynthate into the soil. Thus, the biological aspect of that portion of the carbon cycle represented by microbial mineralization has modified the physical environment so as to increase carbon fixation by the plants, which in turn allows for further enhancement of the soil microbial populations dependent on generation of this plant carbon. This portion of the ecosystem development could be considered to be a true synergistic interaction.

The interdependence of higher plants, microbes, and their physical environment (not simply from a fixed environment, but more from the view that these interactions are resulting in a continuously evolving ecosystem) is demonstrated by the simple example of site management noted above. Similar interactive effects can be exemplified by evaluating the biological and chemical interactions involved with acid mine drainage-impacted soil. Many of the ores mined throughout the world are sulfide salts (e.g. iron sulfide and zinc sulfide). As long as the ecosystems containing the sulfide salts are not disturbed, the conversion of the sulfides to sulfates is limited by the anoxic or very low oxygen levels. Mining of the metal ores results in oxygenation of the metal sulfides to more water-soluble metal sulfates. This process results in acidification of the soil sufficiently that the indigenous acid-sensitive microbial population ceases to function. Reclamation of the soil requires raising the pH sufficiently that biological activity precluded by the acid may be resumed. Since biological sulfate reduction to sulfide requires anoxic conditions, site management must include flooding of the soil to encourage development of sulfate-reducing bacteria. The ameliorative processes eventually result in reestablishment of biogeochemical cycling of nutrients. (See Mills (1985) for a further discussion of management of acid mine drainage.) The microbial role in

this situation simply involves chemical reduction of the soluble sulfate salts to sulfide, i.e. the sulfate serves as a terminal electron acceptor for the microbial community, which results in precipitation of the metal sulfide under the anoxic conditions. Note that the anoxic conditions must be maintained to prevent the soil microbes from oxidizing the sulfide to sulfate as a metabolic energy source.

These examples demonstrate the optimization of microbial community development which results from modification of the physical and chemical environment due to the action of the biological components. Traditionally, this "optimization" is generally considered to entail improvements in community structure, which translates from an anthropogenic viewpoint into an esthetic aboveground community. However, ecosystem evolution may involve alterations in the chemical environment that cause declines in aboveground productivity. For example, drainage of acid peats (either artificially or anthropogenically induced) stimulates the microbially catalyzed oxidation of the metallic sulfides contained therein into sulfate. The resultant decline in soil pH results in death of acid-sensitive plants in the aboveground community.

Biological cycling of nutrients in soils causes both permanent (long-term) and transient (seasonal) alterations of the physical environment. Solubilization of minerals and their subsequent movement into plant tissue or even leaching from the soil site result in an irreversible change in soil mineralogy. In contrast, more ephemeral changes in soil components are associated with the interaction between the aboveground plant community and soil microbes. For example, total system fixed nitrogen loads may be increased through the activity of nitrogen-fixing bacteria. This nutrient augmentation results in a general improvement in soil fertility. The effect may be limited if the newly incorporated fixed nitrogen is removed from the site (e.g. harvesting of crops, anthropogenically or by grazing animals), leached from the system in percolating waters, or denitrified.

The preceding analysis shows that both microbial and plant communities benefit from biogeochemical cycles. This gain by the biological community is enhanced by the associated modification of the physical environment by soil microbes. It must be stressed that no volition on the part of the communities or individual organisms is assumed in these adaptations. Beneficial alterations of the organisms' surroundings are purely fortuitous. A situation is created wherein the microbial communities alter their habitat in a manner that results in selection of more effective microbial populations. This interaction produces a continuously evolving ecosystem.

9.2.2 Interconnectedness of Biogeochemical Cycle Processes

Commonly, the best way conceptualizing the biogeochemical cycles is to examine them as independent entities, as has been the historical educational practice. Yet, to some degree this presents an impediment to developing an absolute appreciation of the intricacies of nutrient cycling in the soil system. Central to all nutrient cycles are

the various ecosystem components composed of organic matter – a variety of plant, animal, and microbial biomass pools plus intermediates in the mineralization of these components along with soil humic substances. This organic matter-centered concept of biogeochemical cycling is depicted in Figure 9.5 (where the assembly of

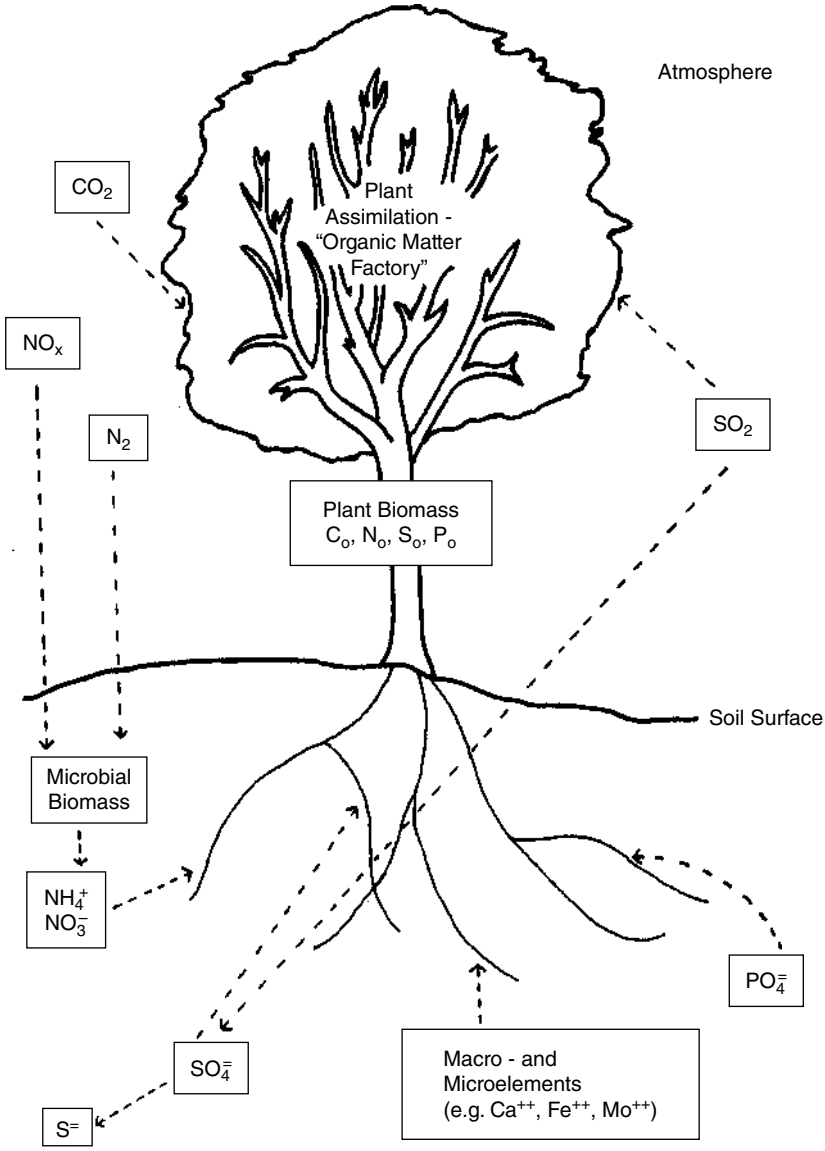

Figure 9.5 Examples of the central role of plant biomass in sequestering various nutrient elements. (The subscript "o" refers to various organic pools of the individual element.)

the components of biomass is shown) and Figure 9.6 representing the disassembly or mineralization of organic substances to the primary mineral pools.

The pivotal role of organic matter in biogeochemical cycles results primarily from the fact that it is the primary reservoir of carbon, oxygen, nitrogen, phosphorus, and sulfur in soil. Thus, in an unmanaged system, the movement of nutrients into this organic matter reservoir and their subsequent mineralization is the primary controller of plant nutrient availability. A conceptual model accentuating this fact is shown in Figure 9.7. The primary components of biomass can be envisioned as

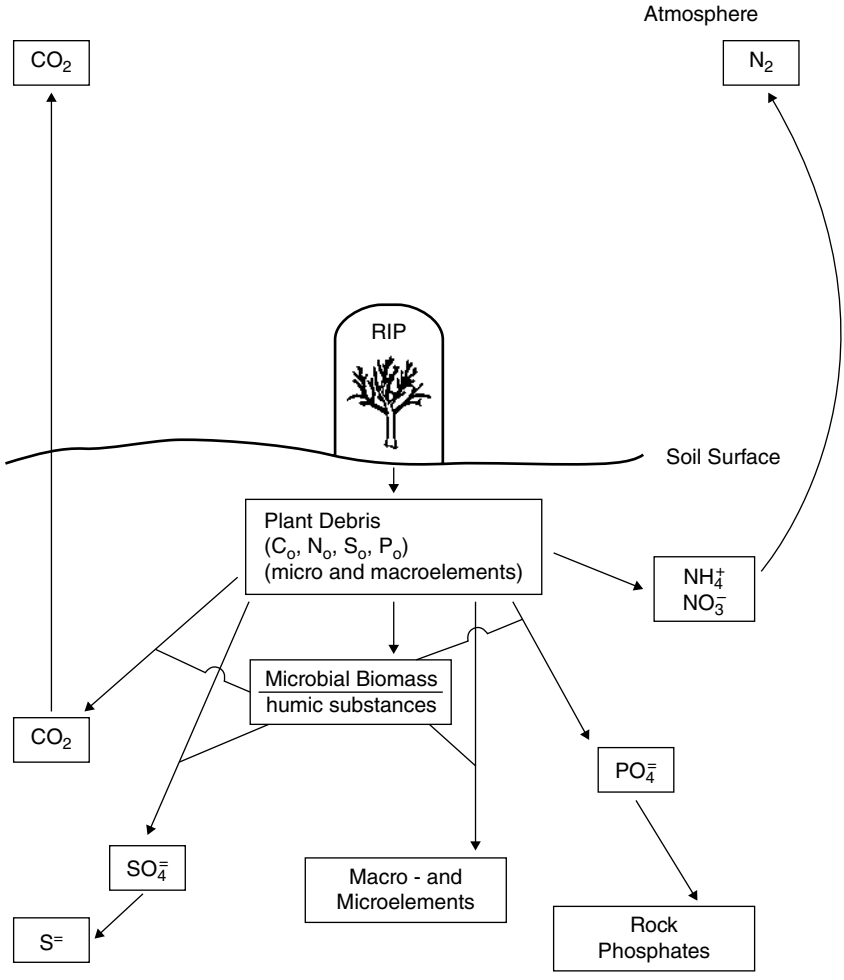

Figure 9.6 Disassembly of plant organic matter into various primary inorganic nutrient pools. (The subscript "o" refers to various organic pools of the individual element.)

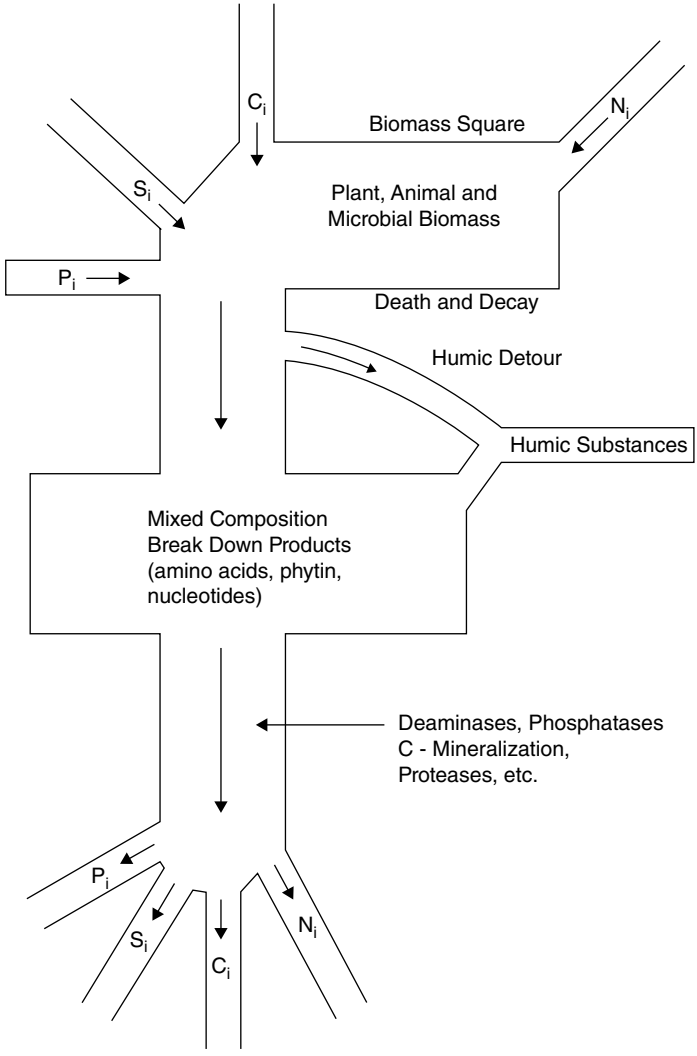

Figure 9.7 The road from an inorganic existence through biomass and return to inorganic forms. The subscript "i" refers to inorganic forms of the element.

traveling along a highway that converges on a "square" consisting of living cells. Some organic substances are lost from the biomass and exit the square as biomass products (e.g. root exudates, extracellular enzymes), but most enter the "decomposition lane" upon demise of the cell. Some of the organic substances take a detour through the neighborhood of humic substances, but eventually all enter a square consisting of mixed composition organic compounds, e.g. amino acids, nucleotides,

carbohydrates, phytin, and humic substances. Continued mineralization of these products yields the original mineral components.

A practical difficulty resulting from this interaction of the biogeochemical cycles is the designation of a particular mineralization process as being driven by the needs of an individual nutrient cycle. For example, amino acid mineralization in soil can be easily quantified. Major products of the process are cellular energy, carbon dioxide, ammonium, and sulfide. A question that could be considered is whether the ammonium is produced because of a requirement of the decomposer population for ammonium to synthesize new biomass or whether the mineral nitrogen was produced as a by-product of the carbon and energy recovery processes. In fact, mineralization of carbon and nitrogen are sufficiently linked in soil that assessment of carbon dioxide evolution rates has been suggested as a means of estimating net nitrogen mineralization (Gilmour et al. 1985). The bottom line is that it is easy to produce models descriptive of various nutrient cycles, but difficult to designate particular processes as being primarily driven by needs of any specific nutrient cycle.

9.3 Biogeochemical Cycles as Sources of Plant Nutrients for Ecosystem Sustenance

A good way of gaining an appreciation of the interactive nature of the aboveground and belowground portions of the ecosystem is to examine the nutrient cycling interactions between the two regions. Nutrients enter soil through rainfall, dry deposition (e.g. sulfate, nitrate, and minerals contained in dusts), atmospheric gases (e.g. sulfur dioxide), anthropogenic sources (e.g. soil amendments), and fixation (dinitrogen, carbon dioxide). Primary sources of the basic building blocks of organic matter are divided between the atmosphere (carbon dioxide and dinitrogen) and soil (phosphate- or sulfate-bearing soil minerals). Sulfur sources could be considered to be divided between soils (sulfur-bearing minerals) and atmospheric sources (e.g. sulfur dioxide generated by power generation and, to some extent, automobile exhausts). With activities directed at alleviating air pollution, especially from automobiles and power plants, the latter source of sulfur compounds is being reduced, which has resulted in a need to consider soil mineral and organic matter derived sulfur inputs for plant biomass productivity.

As an ecosystem matures (approaches equilibrium), reliance on external nutrient inputs declines. The majority of the materials necessary for biomass productivity are produced by mineralization of resident organic matter pools. This situation is most clearly seen with nitrogen cycling in a mature forest. In a climax forest, the vast majority of the nitrogen is contained in soil organic matter and plant biomass nitrogen pools (e.g. Tate 1987). In these situations,

nutrient availability to the plant community is reliant nearly totally on the rate at which the microbial community mineralizes plant, animal, and microbial biomass as well as soil humus.

Therefore, in ecosystems receiving minimal inputs of nutrients, a truncated nutrient cycle could be said to exist. The various nutrients are cycled between organic and mineral nutrient pools. Organic nutrient resources are composed of plant biomass and photosynthate entering the soil and native soil organic matter. These organic matter pools can be separated into (i) those substances delineated on the basis of solubility (humic acids, humin, and fulvic acids), and (ii) recognizable biochemical components of living cells; that is, polysaccharides, amino acids, nucleotides, etc. The latter substances are usually classed as readily available organic matter (see Chapter 10 for a discussion of the importance of this reservoir of organic material in ecosystem productivity). Readily available organic matter is that portion of the soil organic matter reservoir that is easily metabolized by the soil microbial community. It is distinguished by its short residence time (generally less than 1–2 years) and the fact that it provides essentially all the metabolic energy for the soil microbial community.

Ecosystems may also derive a major portion of their energy from externally supplied organic substances. These situations are usually the product of anthropogenic inputs. Specific examples are intensively cultivated agricultural soils, where crop debris and anthropogenically produced fertilizers provide the bulk of plant nutrients, plus soils impacted with varying inputs of sludges and composted materials. Soil amendment with these societal residues may be significant plant nutrient sources (e.g. see Boyle and Paul 1989; Geiger et al. 1992; Holland and Coleman 1987).

9.4 General Processes and Participants in Biogeochemical Cycles

The soil biological community mobilizes minerals, mineralizes organic substances, and immobilizes nutrients directly through a combination of oxidation and reduction reactions or indirectly through environmental modification. Direct reactions may involve alteration of the oxidation state of the particular element of interest as well as oxidation or reduction reactions. For example, energy recovery and mineralization of carbon-based compounds involve oxidation (electron removal) of the carbon compounds to carbon dioxide. Similarly, ferrous iron may provide energy for microbial growth by removal of electrons to produce ferric iron. Alterations of the oxidation or reduction state are associated with oxidation of ammonium to nitrate and sulfide to sulfate. In each of these instances, the energy yielded by the oxidative electron transfer produces increases in microbial biomass.

Lest a false impression be created regarding the direction of the oxidation–reduction processes in soil, it should also be noted that a variety of reductive transformations are essential for integrity of the biogeochemical cycles. For example, whereas organic compounds are being oxidized by denitrifying bacteria, the electrons so generated are utilized to reduce nitrate to dinitrogen. Similarly, sulfate may also serve as an electron acceptor – producing hydrogen sulfide among the products – in accompaniment with oxidation of a variety of organic carbon compounds.

Minerals may also be solubilized within the ecosystem by less direct means than direct alteration of their redox state by actively growing microorganisms. For example, chelators solubilize metals and modification of the microsite pH may result in solubilization of a variety of cations. Acidification results in solubilization of metals such as aluminum as well as dissolution of carbonate rocks, thereby freeing associated cations.

The primary mediators of these processes are the soil bacterial, actinomycete, and fungal communities. Most research has historically been associated with these groups of organisms because they are responsible for catalyzing the bulk of the reactions involved in biogeochemical cycling. However, recall from Chapter 7 that the activity of these organisms in soil is controlled in part by their interactions with soil protozoa, nematodes, and microarthropods as well as a variety of soil animals (earthworms, ants, millipedes, and insects). Also, although the contributions of these latter biological groupings to nutrient cycling may be less than those of the primary decomposers, the higher members of the soil community also participate in mineralization of organic matter through the respiratory processes necessary to maintain their own biomass. For example, the interactions of protozoa and higher animals in soil are key to mineralization and cycling of nutrients through a variety of means.

- These organisms are instrumental in the mixing of organic matter within the soil profile, thereby augmenting the contact between the plant residues and the decomposer populations. Key participants in these processes include earthworms, ants, termites, and even burrowing animals to some extent.
- Soil animals are involved in inoculation of plant litter with propagules of decomposers. Movement of ants, termites, and other insects between soil nests and surface litter insures contact of active microbial cells with plant debris.
- The soil animals indirectly stimulate decomposition by modifying soil properties. For example, increased aeration results from formation of earthworm galleries, burrows and chambers, and anthills.
- The soil animals may increase the surface area of the organic debris indirectly through their movement through dry deposits of plant biomass as well as directly via chewing and tearing of plant parts. Increased surface area augments potential contact with decomposer populations.

- As indicated above, the organisms may mineralize the plant biomass directly. For example, the woodlouse (*Porcello scaber*) feeds on decaying pine needles (Soma and Saito 1983).
- Trophic interactions as described in Chapter 7 also stimulate decomposition. Interactions between the primary decomposers (bacteria and fungi) with population-controlling nematodes, protozoa, and mites are key in maintaining active primary decomposer populations.

In summary, all soil decomposer populations, primary plus secondary decomposers, derive energy and reducing power (i.e. everything necessary for formation of a stable community) from the transformation of organic and mineral intermediates of the various nutrient cycles. Indirectly, the products of the decomposer populations benefit aboveground populations – plants directly and animals indirectly.

9.5 Measurement of Biogeochemical Processes: What Data Are Useful?

Design of field and laboratory experiments for the assessment of biogeochemical processes is complicated by the generally prevalent desire to extrapolate the data from the level of the soil sample contained within a test tube to an ecosystem-wide or even whole-Earth view. Questions impinging on validity of data extrapolations include considerations of the inclusiveness (or appropriateness) of the data collected and the selection of representative soil samples. Furthermore, manipulation of the soil sample (i.e. storage conditions, mixing, air drying) subsequent to collection may preserve or alter the relationship of process rates occurring in the sample from those occurring in the field.

9.5.1 Assessment of Biological Activities Associated with Biogeochemical Cycling

As was introduced in Chapter 2, a variety of methods are available for assessing biological activity in soil samples. Furthermore, a multitude of assays of enzymatic activities, which are useful for the estimation of transformation rates associated with various biogeochemical processes (e.g. dehydrogenase, phosphatase, as well as assessment of the oxidation rates of a variety of carbonaceous substrates, such as glucose and aromatic compounds), have been adapted for use with soil samples. Applicability of the laboratory assessments of soil systems to the field situation is controlled by inherent limitations of the procedures themselves as well as by difficulties associated with provision of a meaningful extrapolation of the results beyond the individual soil sample studied.

Problems with quantifying biological activities in soil for determination of nutrient cycling potential and rates can be exemplified by evaluating applicability of various measures of soil microbial biomass. Several methods have been described for estimating microbial biomass levels and activity (e.g. respiratory activity [oxygen uptake or carbon dioxide evolution] as well as dehydrogenase activity, chloroform fumigation, ATP measurement, PCR analysis, and direct examination). See Chapter 2 for a discussion of each of the methods. Considerations associated with utilization of each procedure involve the intrinsic limitations of the assay (i.e. what is and is not measured) plus variability of microbial biomass densities between soil samples and within a soil site.

For any scientific procedure, the researcher must specify what the assay is truly measuring. For example, it is easy to postulate that carbon dioxide evolution rates from soil are proportional to the activity of the decomposer population in that soil sample, without realizing that carbon dioxide is also produced by those organisms feeding on the primary decomposers plus any plant root tissue in the sample. Thus, such data likely yield an overestimation of carbon mineralization in an ecosystem. Similarly, it is reasonable to consider that the total active soil microbial biomass would be related to carbon mineralization potential in a soil sample, but microbial biomass assessment techniques – chloroform fumigation, ATP measurement and direct counts – all result in the quantification of both active and inactive microbial propagules.

9.5.2 Soil Sampling Aspects of Assessment of Biogeochemical Cycling Rates

Data interpretation is also complicated by the fact that the assays are generally conducted in the laboratory. Assuming that a representative field soil sample may be collected, data from analysis of the sample in the laboratory may not reflect actual population densities or activities in the field. A variety of changes in the soil properties generated by removal of the sample from its native site, its storage conditions, and preparation for study may result in alteration of the biological activity occurring therein. For example, collection of the soil sample as well as any sieving or mixing of the soil that occurs in the laboratory releases native soil organic matter trapped within soil aggregates. At least a portion of this organic matter can be mineralized by the microbial community. Thus, the biomass and the enzymes required for catabolism of the organic matter may be augmented above levels previously existing in the field. This complication is increased if storage of the sample is necessary. Even at refrigerator temperatures, significant growth of microbial populations can occur. Some enzymatic activities are preserved at reduced temperatures, whereas other activities are not (see Tate 1987 for a more complete discussion of this topic). Therefore, for any measure of biological

activity in a field sample, control experiments must be conducted to determine stability of the variable of interest during storage.

Extrapolation of laboratory-generated data to the field from which the sample was collected is reliant upon the sample being representative of the field situation. This prerequisite for data analysis is nowhere more essential than in studies of biogeochemical cycles. With few exceptions, laboratory assays are conducted with soil sample sizes ranging from less than 1 g to perhaps 100 g at most. As indicated in Chapter 1, horizontal and vertical variation of microbial populations in soils as well as the containment of microbial populations within discrete microsites within the soil matrix makes collection of manageable soil samples that are representative of an entire field or ecosystem site difficult. To produce data in the laboratory that are at least partially representative of the field situation, care must be taken to collect a series of soil samples that represent pertinent portions of the ecosystem of interest. This representative soil sample must then be preserved in a manner to minimize or preclude alteration of biological activities prior to their quantification.

9.5.3 Environmental Impact of Nutrient Cycles

As indicated above, biogeochemical cycles are composed of a variety of chemical compounds, linked in a cyclic manner, whose interconversion is accomplished by a combination of biological and chemical processes. Although a mass of data has been published documenting the size of various nutrient pools within a number of ecosystems, considerable interest must be directed at the rate of movement between these pools and the environmental impact of these transformations. Alteration of the distribution within the various pools or their rate of change will necessarily alter the ecosystem and adjacent system function. Frequently, complications arise from the failure to fully consider inputs or losses from the soil system. For example, nutrients may leave a system through leaching, denitrification, volatilization (including ammonium volatilization) as well as being lost in harvested crop. The latter category is not limited to that biomass removed through agricultural processes. Wild and domestic animals may be prime movers of nutrients from an ecosystem through their grazing activities. Positive as well as negative system impacts are easily encountered.

This movement of nutrients between various reservoirs has been exploited for agricultural crop production. “Mining” of fixed nitrogen contained in soil humus has long been the basis for successful subsistence agriculture, but this enhancement of nutrient transfer from organic to mineral reservoirs is not limited to low-input, limited harvest cropping systems. Histosols, organic soils, are also so exploited. Peats and swamps represent sidetracks in nutrient cycles. Carbon, nitrogen, phosphorus, and sulfur compounds are incorporated into plant biomass

which due to waterlogged conditions may be preserved for thousands of years. Removal of impediments to microbial mineralization of the organic matter results in rapid mineralization of the biomass. This produces sufficient nitrogen to support an intensive agricultural cropping system, as exemplified by the Everglades Agricultural Area (South Florida). Decomposition of the organic matter comprising the muck soils of that region produces as much as 3.3×10^4 kg C ha^{-1} year^{-1}. This is accompanied by production of approximately 1400 kg N ha^{-1} year^{-1} (Tate 1980). For comparison, soils of a tropical rainforest are estimated to produce 405–2117 g C m^2 year^{-1} (Schlesinger 1977). The above carbon evolution rate for the histosols in southern Florida is equivalent to 3300 g C m^2 year^{-1}.

In undisturbed ecosystems, such as mature forests, mineralization and biomass synthesis rates are nearly balanced. That is, recovery of the mineral nitrogen produced by the soil microbial community through plant roots is sufficiently efficient that little nitrogen leaves the site through such processes as leaching to groundwater. These slight losses due to percolation of mineral nutrients to ground or through surface water flow are balanced by nitrogen inputs due to nitrogen fixation. Concerns arise from those systems, especially disturbed sites, where nitrogen leaching may reach ground or surface waters (such as the movement of nitrate and phosphates into Lake Okeechobee due to oxidation of the histosols of the Everglades Agricultural Area). Economic considerations arise when externally supplied nutrients (fertilizers) added to agricultural soils leach from the system rather being retained in the crop.

9.5.4 Example of Complications in Assessing Soil Nutrient Cycling: Nitrogen Mineralization

Nitrogen mineralization rate is a key indicator of the potential biomass production that can be supported by indigenous mineral nitrogen production. As indicated above, the vast majority of the nitrogen contained in soils exists as organic nitrogen. Thus, in the absence of exogenous inputs, new biomass cannot be synthesized without mineralization of the resident organic nitrogen pools. The size of these nitrogen pools (organic and mineral) and flux rate between them are therefore meaningful values in characterization of an ecosystem.

Two procedures most commonly used to measure soil nitrogen mineralization potential are (i) the Stanford and Smith (1972) procedure, which was originally proposed for the study of soils contained in a column but has also been used for soils incubated as a batch sample in a beaker, plus (ii) the buried bag method (Eno 1960; Pastor et al. 1984). With the Stanford and Smith method, soil samples are collected and transported to the laboratory where they are sieved to increase homogeneity and to remove plant debris. The prepared soil samples are then

dispensed into soil columns (leached method) or distributed into beakers (batch method). After an appropriate incubation period, mineral nitrogen is extracted from these samples with a dilute $CaCl_2$ solution. Nitrate plus nitrite and ammonium production are assayed over a time frame usually varying from two to eight weeks. With the buried bag procedure, soils are collected in the field and placed either with minimal manipulation or following sieving into a polyethylene bag. The bag is returned to the soil horizon in a position as closely replicating the situation of the collected soil as possible. In theory, the conditions within the bag represent the field situation except that free exchange of soil water is prevented. That is, it is usually assumed that gaseous conditions within and without the bag are equivalent. Data from Bremner's laboratory (Bremner and Douglas 1971) indicate that free oxygen concentrations may decline inside the bag to levels that would reduce the activity of aerobic microbial populations. For an example of the use of these two procedures with forest soils, see Poovarodom and Tate (1988) and Poovarodom et al. (1988). With each procedure, mineral nitrogen production rates are determined by quantifying ammonium and nitrate plus nitrite concentrations in the soil samples before and following incubation.

The primary objective of conducting these assays is to measure the quantities of organic nitrogen that can be mineralized (size of the nutrient pool) and how fast it is being mineralized (flux rate). Note that these two values are not directly measured by the above procedures. Although direct assessment of the size and mineralization rate of labile soil organic nitrogen pool would be ideal, due to the complexity of the soil organic nitrogen pool, this is impossible. Soil organic nitrogen compounds are a highly diverse mixture of compounds, only a portion of which is easily metabolized by the soil microbial community. Furthermore, no soil organic nitrogen component has been found to be directly proportional to the mineralization rates. Therefore, these values must be derived indirectly. Both the quantities of mineralizable nitrogen contained within a soil sample and its mineralization rate are calculated from nitrogen mineralization data. Such data are predicated upon the assumptions that (i) soil organic nitrogen mineralization occurs in the laboratory-incubated or buried bag samples at rates comparable to the field situation, (ii) preparation of the soil samples for analysis does not alter the availability of the organic nitrogen to the microbial community, and (iii) mineralization is a first-order process occurring at the same rate throughout the incubation period.

Determination of the nitrogen mineralization rate (Stanford and Smith procedure as well as buried bag technique) is usually predicated upon the observation that nitrogen mineralization follows first-order kinetics:

$$dN / dt = -kN \tag{9.1}$$

where N is the concentration of mineralizable organic nitrogen, k is a rate constant and t is time. Integration of this equation produces the following:

$$N_t = N_O e^{-kt} \tag{9.2}$$

where N_t is organic nitrogen at time t and N_O is organic nitrogen at time zero. Since the nitrogen at time t is equivalent to the initial nitrogen minus the nitrogen mineralized (N_m), the equation can be reduced to one containing a single unknown:

$$N_t = N_O - N_m \tag{9.3}$$

$$N_O - N_m = N_O e^{-kt} \tag{9.4}$$

$$N_m = N_O \left(1 - e^{-kt}\right) \tag{9.5}$$

These calculations provide an estimate of the original organic nitrogen and its mineralization rates under the conditions where the data were collected (i.e. the laboratory or within the buried bag). Thus, the derived parameters are not necessarily applicable to true field situations. For example, N_O represents the initial nitrogen concentration in the disturbed soil sample; that is, the nitrogen available to the microbial population after the aggregates were disrupted by sampling and sieving of the soil sample. A significant proportion of that nitrogen could have been trapped in soil aggregates prior to sampling. Thus, the mineralization rate derived from the laboratory-incubated and buried bag retained soils may be an overestimation of that occurring in the field.

Further inaccuracies in the data derived by this method are added by the potential variation in nitrogen production due to diminished oxygen availability in the buried bag procedure (and the potential for this to occur in batch-incubated samples), which would result in an underestimation of $N_{m.}$ Complications associated with the Stanford and Smith leaching procedure involve the removal of mineralizable organic matter during leaching of the columns (Smith et al. 1980). With the latter nitrogen mineralization assay method, the soil columns are leached with a salt solution, prior to and at intervals during the incubation time. The resultant leachate contains the soil mineral nitrogen, which is the objective of the leaching procedure. It also includes a portion of the water-soluble organic nitrogen. Removal of this latter soil nitrogen fraction from the pool of organic nitrogen may reduce the actual mineralization observed during the incubation procedure. Each of these difficulties in incubation of the soils – modification of soil atmosphere and removal of mineralizable organic nitrogen – may alter the rate of ammonium, nitrate, and nitrite production by the soil microbial community. Thus with all of these procedures, at best, an estimate of the field-available organic nitrogen pool and its mineralization rate is provided.

The model described above is based on observations that nitrogen mineralization in soil generally follows first-order kinetics. Other mathematical models for deriving N_O and k have been proposed that are based on alternative nitrogen mineralization kinetics (e.g. Bonde and Lindberg 1988; Broadbent 1986; Cabrera and Kissel 1988; Deans et al. 1986; Juma et al. 1984), but the first-order model described above appears to be the most commonly used for derivation of these nitrogen mineralization constants.

These observations demonstrate the degree of difficulties that are encountered in producing data representative of field nutrient pool size and the flux between these various pools. Such considerations of cycles where large mineral pools exist are complicated by difficulties in assessing small changes in mineral nutrient against a large background. This problem is commonly encountered with measurement of fluxes of organic phosphate and sulfate against backgrounds of soil-soluble and rock phosphate. These observations clearly reveal the basis for problems with ecosystem-wide and global extrapolation of such data.

9.6 Conclusions

An appreciation of the properties of the reactions occurring in biogeochemical cycles and the impact of variation in environmental factors on their reaction rates is essential for a complete understanding of a native ecosystem and in development of soil management or reclamation plans. These cycles are commonly appreciated solely for their contributions to aboveground plant productivity and their impact on ecosystem development, but it must be understood that biogeochemical processes are much more complex than the simple biological or chemical catalyzed transformation of an essential plant macro- or micronutrient. A complete depiction of biogeochemical cycles must entail an understanding of not only the biological entities involved but also their synergistic interactions with the soil physical and chemical properties as they contribute to development of the native ecosystem. For reclaimed soils, this synergism impacts development of a self-sustainable ecosystem.

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10

The Carbon Cycle

Conceptually, models of the carbon cycle can easily be reduced to representations of the processes associated with the transformation of carbon dioxide into organic matter and return of this primarily photosynthetically fixed carbon to its mineral form via biological mineralization (see Figure 9.1). This simplistic depiction of the carbon cycle processes provides a means for developing generalized principles, but amassing the knowledge necessary for managing soil sites for ecosystem sustenance or reclamation is reliant upon separation of these two primary carbon reservoirs into pools designated by location in the ecosystem, residence time, and/or chemical form. Commonly studied soil organic carbon reservoirs may include living and dead microbial biomass, animal and plant debris, plant root exudates, as well as humic and fulvic substances (Figure 10.1). Even this grouping of soil organic components must be expanded conceptually before a detailed understanding of the properties of both the individual compounds themselves and their interactions with ecosystem components that limit the rate of flux between these organic reservoirs and the mineral pools can be presented. A reasonably detailed understanding of (i) the quantities as well as the properties of the biological residues (e.g. protein, polysaccharide, lignin, fat, waxes, and resins) and those substances formed via chemical or a combination of biological and chemical reactions (humic substances) entering or contained in the various soil organic matter reservoirs, (ii) the transformations occurring both within living biomass as well as external to the cell structure that impact the fate, long term as well as short term, of organic matter in soil, as well as (iii) the speciifc biological and chemical process that control the retention and loss of soil organic matter is essential for detailed modeling of the soil carbon cycle.

Some soil organic matter pools can be distinguished by their degradation resistance or vulnerability (e.g. humic substances vs animal or plant biomass) but most of the organic matter pools designated in Figure 10.1 are mixtures of biodegradation-susceptible and relatively stable organic components. Furthermore, due to

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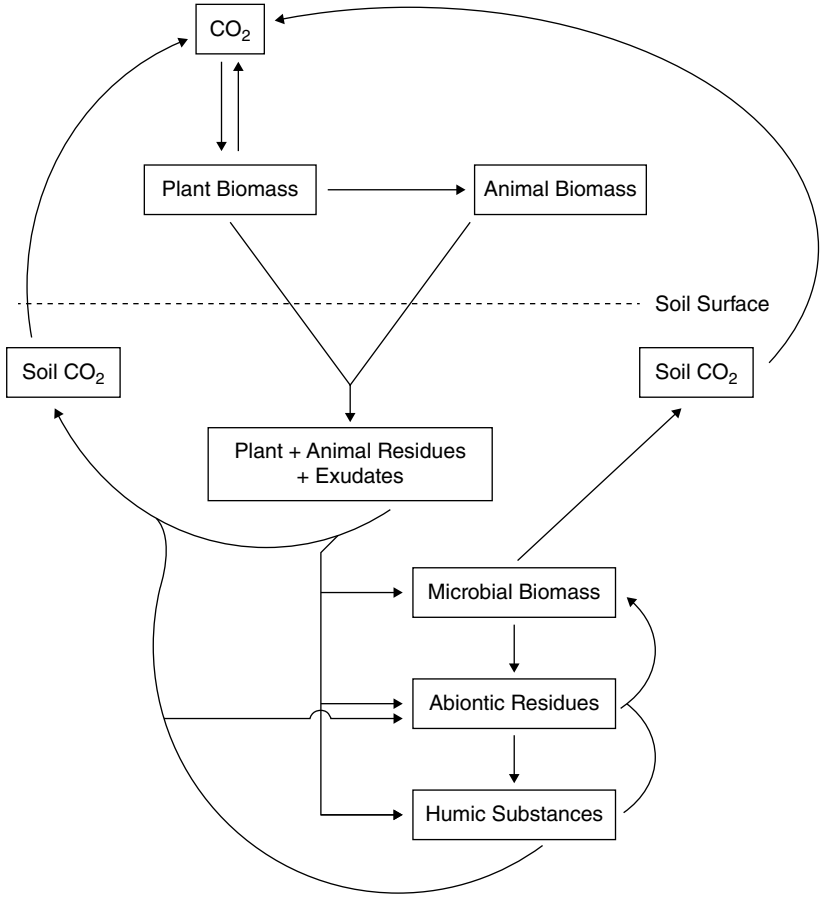

Figure 10.1 Conceptual model of carbon cycle with transfers between major soil organic matter reservoirs emphasized.

the occurrence of complexes of mineral and decomposable organic matter and the stability of plant cell structure, the decomposition kinetics of a portion of the easily metabolized substances reflects those of the more resistant materials. Consequences of interactions within the soil environment are exemplified by an analysis of plant biomass decomposition in soil. Accessibility of easily decomposable plant biomass components to the soil microbial population is controlled at least in part by the rate of breaching of the plant cell wall. Biodegradation of readily metabolized organic compounds is also reduced by their occlusion in soil aggregates. Thus, estimates of the rates of mineralization of easily decomposable carbon pools must encompass the degree of enhancement of their stability by

inclusion in soil aggregates and stabilization of plant cellular structure. Similarly, a normally decomposable xenobiotic compound, such as some pesticides, may be extended by its sorption to soil mineral particles (e.g. clays) or of associations with soil humic substances.

In conclusion, to develop a clearer understanding of the complexity of the processes leading to increases or decreases in quantities of sequestered organic carbon in soil, the nature of the components of this soil fraction and the processes leading to their decomposition must be elucidated. Each of the major organic matter reservoirs in soil is a mixture of readily metabolized organic compounds plus relatively biodegradation-resistant substances, such as lignin and humic acids. Biodegradation-resistant humic substances constitute a major portion of the global carbon stores in soil. In contrast, the size of the pool of easily metabolized resources determines the quantity of energy available to drive the soil microbial population and "propel carbon through the carbon cycle." Ramifications of both of these processes relate to both function and stability of individual soil systems as well as to extended global concerns, such as the role of soil ecosystems in mediation or augmentation of greenhouse gas production and consumption. Therefore, this chapter has the objectives of (i) evaluating the nature of organic compounds retained within major soil organic matter reservoirs, (ii) elucidating the mechanisms involved in the catabolism of these compounds, and (iii) determining the properties of the carbon molecules, the microbial community, and the soil ecosystem itself, which are instrumental in controlling the return of soil organic matter to atmospheric carbon dioxide pools.

10.1 Environmental Implications of the Soil Carbon Cycle

Considering our ability to provide nutrients required for plant growth, ameliorate the impact of toxic materials (such as strong acids or plant toxic materials) found at the site of cultivation, and to institute plant growth-favorable physical and chemical conditions, it is reasonable to conclude that, with our current state of knowledge, a plant community can be established on nearly any soil-like supportive matrix. This conclusion is based on the assumption that unlimited human intervention is feasible to support the resultant high-maintenance production system. Note that this conclusion is based on the postulate that ample energy resources are also available. Highly artificial plant growth systems can be maintained at high expense but a sustainable, fully functional ecosystem is reliant upon occurance of nutrient cycles throughout the soil system. At the top of the list of essential soil processes needed to support a stable biological community is a carbon cycle.

Studies of organic matter fluxes occurring in specific soil reservoirs are frequently inspired by a concern for such ecological and societal problems as demonstration of the potential for utilizing soil systems to recycle organic carbon-based wastes, reclamation of sites impacted by mismanagement and, from a less direct viewpoint, the impact of soil carbon mineralization and assimilation processes on global processes such as climate change. Historical interest has also been centered on the determination of best management practices for enhancement of soil quality and optimization of ecosystem productivity. For example, data from studies of carbon cycling in soil systems are needed to suppport decisions involving the management of greenhouse gas fluxes, maintenance of natural ecosystems, disposal of societal wastes, reclamation of degraded soils, and the enhancement of agricultural production of food and fiber.

10.1.1 Soils as a Source or Sink for Carbon Dioxide and Methane

Soil ecosystems are producers and consumers of the ultimate end-products of carbon mineralization – carbon dioxide and methane - both of which are major contributors to global warming. Sequestration of these gases in plant biomass and soil organic matter is a process that can be controlled, at least to some degree, via appropriate soil management for reduction of greenhouse gas production and appropriate management of soil properties so that optimized assimilation of carbon dioxide into plant biomass reduces the amount of the greenhouse gas lost to the atmosphere. Both carbon dioxide evolution and sustained plant growth are soil properties that can be controlled through optimized site management. Increases in soil organic matter contents can result from sequestration of carbon, thereby reducing the quantities of soil carbon entering the atmosphere. That is, with appropriate management, soils can become a net pool for carbon dioxide. Causing declines in colloidal soil organic matter (e.g. via intensive cultivation) converts the soil system into a source of greenhouse gases.

This observation leads to the conclusion that increases in soil organic matter pools and the optimization of plant biomass productivitiy can be major means to optimize the amount of carbon sequestered in plant biomass and soil organic matter, thereby reducing the quantities of carbon dioxide evolving from soil.

An example of soil system management that can result in significant increases in soil organic matter resources is conservation tillage. Kern and Johnson (1993) estimated that implementation of no tillage cultivation could result in sequestering of carbon in soil organic matter equivalent to 0.7–1.1% of the total projected US fossil fuel carbon emissions of a 30-year period. A less direct but significant interaction between soil organic matter reservoirs and atmospheric mineral carbon concentrations results from management of soil properties for optimization of plant biomass production (i.e. ecosystem productivity).

Basically, increases in soil organic matter pools and the optimization of plant biomass productivity (two major soil carbon pools) can meaningfully impact global climate change. Looking a little closer at the processes occurring here, an interacting association between atmospheric processes, plant community development, and soil minerals is revealed. This benefit of the association is a product of their interaction – soil particle aggregation. Plants utilize sunlight for energy to produce biomass, a portion of which, upon death of the plant, is incorporated into soil where it provides energy for growth of soil microbes. The soil microbes produce polysaccharides that contribute to the forces binding soil particles together into soil aggregates. The product of this is improved soil structure, which in turn allows for greater plant growth (see Chapter 1 for more details on these processes).

Since the terrestrial biosphere and associated soils contain far more carbon than the atmosphere, these carbon pools may serve as buffers or modulators of atmospheric carbon dioxide levels. For example, soil organic matter and terrestrial biomass most likely account for at least a portion of this unknown fate of carbon dioxide (e.g. Harden et al. 1992; Sundquist 1993). An estimate of mass of carbon in various biogeochemical pools is provided by Sundquist (1993), where it is estimated that 750 Gt (1 Gt = 10^{15} g) of carbon was contained in the atmospheric pool in 1990 CE compared to 2160 Gt for the terrestrial biosphere, with the soils component accounting for approximately two-thirds of this total. Eswaran et al. (1993) estimated that 1576 Gt of carbon is stored in soils, with about 32% of this carbon retained by tropical soils. The importance of soil organic matter as the primary carbon pool is exemplified by considering the distribution of carbon in a forest soil. It has been estimated that between two- and threefold the carbon retained in forest biomass is found in dead organic matter on the forest floor compared to atmospheric carbon dioxide (Kögel-Knaber 1993).

Conversely, the existence of the large biosphere carbon reservoir means that minor changes in biomass production or in retention of fixed carbon within soil can result in significant increases or decreases in atmospheric carbon dioxide levels. That is, biosphere carbon provides a buffer to changes in atmospheric carbon dioxide loads. Potentially world-impacting ecosystem modifications include (i) cultivation of virgin soils (Tate 1987), (ii) drainage of peat soils (e.g. Armentano 1980; Tate 1980) and (iii) clear-cutting of forest biomass and its replacement with less productive plant species.

10.1.2 Diffusion of Soil Carbon Dioxide to the Atmosphere

A further consideration in modeling the exchange rate between soil organic carbon pools and atmospheric carbon dioxide is the diffusion rate of carbon dioxide between soil air and the atmosphere. Variation in more localized soil properties

(i.e. moisture, structure, temperature) scontrol the gaseous exchange rates but also delimit the mineralization rate (i.e. the mass of carbon dioxide available to move from soil to atmospheric pools) characteristic of the soil site (see Oades 1988). The direction of movement is generally from soil air to the overlying atmosphere in that concentrations of gaseous carbon dioxide in soil pores generally exceed atmospheric carbon dioxide loads.

A variety of soil properties interact to facilitate or inhibit this gas exchange. Along with the increases in mass of carbon dioxide resulting from ecosystem modifications listed above, soil moisture, temperature, structure (aggregate formation and pore structure), biological activity (root development and earthworm activity), and mass movement of water (e.g. rainfall) also control carbon dioxide distribution. Less dramatic variability in ecosystem properties than those resulting from ecosystem-wide alterations, i.e. natural and anthropogenically induced, may determine the carbon dioxide exchange rate between soil and atmospheric reservoirs. For example, a minor alteration in soil moisture and temperature, such as those resulting from developing crop canopies, can alter the carbon dioxide flux rate. These interactions were presented in a model developed by Ouyang and Boersma (1992a, b). Additionally, it must be noted that transient increases in soil carbon dioxide levels can alter the composition of the soil microbial community. For example, Kandeler et al. (2008) demonstrated that a transient change in soil carbon dioxide levels resulted in shifts in the fungal community in grassland soils.

Not all carbon dioxide diffusing from the soil surface leaves the ecosystem as a whole. A portion of it can be intercepted by the plant community and fixed into new plant biomass. Increased atmospheric carbon dioxide thus can result in an increase in total aboveground biomass production in established ecosystems (e.g. McMurtrie et al. 1992; Polglase and Wang 1992, Rogers et al. 1983; Wasaki et al. 2005). These increases in biomass production are in addition to the changes in atmospheric carbon dioxide loads due to deforestation and reforestation (e.g. Vitousek 1991; Woodwell et al. 1983). In either situation, enhancement of plant productivity can also alter the equilibrium concentration of organic carbon retained in the underlying soil. Most of the photosynthetically fixed carbon entering soil is mineralized to carbon dioxide by the soil microbial community, but some carbon will be retained in soil in more biodegradation-resistant fractions – microbial biomass and humic substances. Increased fixed carbon inputs may therefore lead to augmented soil microbial populations, their products (e.g. extracellular polysaccharides) as well as greater accumulation of humified substances. Thus, stimulation of biomass productivity by increased carbon dioxide loads could lead to a reduction (albeit of variable magnitude) in atmospheric carbon dioxide and a resultant sequestering of carbon in soil organic matter pools.

10.1.3 Managing Soils to Augment Organic Matter Contents

Due to historical predisposition for the study of soil processes affecting crop yields, most of the data useful for elucidating processes that control soil humus levels are associated with research studies with objectives of improving plant biomass productivity (crop yields). The results of decades of research regarding establishment of natural organic matter levels in soil could be succinctly summarized as follows (see Chapter 1 and Tate 1987 for a more detailed review of this topic).

- The amount of soil organic matter retained in a particular soil system is a product of the balance between organic matter input (primarily aboveground biomass production) and decomposition (predominantly microbial mineralization of plant biomass).
- An equilibrium level of soil organic matter is reached in an undisturbed soil ecosystem. The established quantity of soil organic matter is generally determined by total ecosystem properties. (e.g. the nature of such soil ecosystem properties as the plant community, soil texture, climate, topography, and anthropogenic intervention (see Amundson and Jenny 1991; Jenny 1980).
- Of ultimate importance in evaluation of the impact of soil organic matter transformations on terrestrial carbon cycling is the fact that disturbance of a soil, such as cultivation of virgin prairies or clear-cutting of a forest, results in establishment of a new equilibrium level of soil organic matter generally lower than that in the pristine or undisturbed ecosystem.
- Processes that reduce soil aggregation by increasing soil oxygen levels or soil organic matter availability, such as intensive cultivation or reduction of plant biomass reaching the soil, result in reduced retention of organic matter.

A variety of examples of alteration of soil organic matter resources due to ecosystem disturbance (e.g. clear-cutting of forests, cultivation of forest or grassland soils) and of the relationship of subsequent soil management techniques on establishment of new steady-state organic matter percentages in the soil are available (e.g. Aguilar et al. 1988; Alegre and Cassel 1986; Mann 1986; Rasmussen and Rohde 1988). The extent of change in soil organic matter levels in reaction to ecosystem disturbances tends to be proportional to (i) the amount of disruption of soil structure associated with the initial site clearing, (ii) the extent of cultivation or related management of the soil site subsequent to the initial disturbance (i.e. continued degradation of soil structure and mixing of the soil results in greatest losses of native soil humus), and (iii) the quantities of organic matter produced in or amended to the soil. For example, in the study by Alegre and Cassel (1986), it was shown that in forest soils in the Amazon jungle of Peru, slash-and-burn clearing caused greater retention of native soil organic matter than did mechanical

clearing with a bulldozer. Similarly, maintenance of soil aggregate structure by bedding and lime application reduced organic matter losses compared to that observed in cleared soils that were managed by more structurally damaging procedures. Alteration of inputs of organic matter can be through maintenance of plant productivity (aboveground as well as root mass) and return of *in situ* produced plant biomass to the soil ecosystem (Barber 1979; Richter et al. 1990; Wood et al. 1992) as well as through soil amendment (e.g. for the long-term impact of manure amendments to cropped soils see Jenkinson and Rayner 1977).

Reduction in site intervention, such as that associated with the conversion from intensive tillage to no tillage management of soil, reduces susceptibility of organic matter to microbial decomposition through enhancement of soil aggregate development. See Chapter 9 plus Havlin et al. (1990) and Scott and Wood (1989) for examples of studies of the impact of variation in tillage practices on soil organic matter.

The overall environmental impact of each of these localized soil management procedures is alteration in the quantities of carbon dioxide released to the atmosphere or conversely augmentation of the proportion of total terrestrial carbon residing in the soil organic matter pool. The benefits of the latter situation result in a reduced level of greenhouse gas emissions as well as improved stability and productivity of the ecosystem.

10.1.4 Carbon Recycling in Soil Systems

A major product of human societies is organic waste. These substances include industrial and residential garbage, sewage biosolids, and a variety of petroleum waste products. Incineration of these substances results in conversion of essentially all of the carbon to atmospheric carbon dioxide. Alternatively, utilization of the substances as soil amendments recycles plant nutrients contained therein into plant biomass and allows sequestering of portions of the organic matter into the various soil organic carbon pools.

In the use of soils to recycle organic waste substances, the natural biological decomposers resident in soil are exploited as an economical means of returning bulky, waste vegetative material to less troublesome mineral carbon or stable soil organic carbon reservoirs. For example, see review by Boyle (1990) describing the land disposal of sludge or studies of landfarming of petroleum wastes (see Bossert et al. 1984; Dibble and Bartha 1979a, b; Norris 1980).

Many of the organic components contained in materials such as sludges and composts differ little from plant biomass, litter, and thatch naturally recycled in the soil system. Thus, it can reasonably be concluded that the biochemical processes involved in decomposition of these materials are the same as those associated with general biomass and products of biomass decomposition in soil. Due to differing ratios of decomposable and more biodegradation-resistant components

in the composted materials compared to fresh plant biomass, the kinetics of decomposition generally differ from rates of mineralization of plant biomass. Major concerns with disposal of organic wastes in soil systems commonly are related to (i) the total quantities that can be added before the system is overloaded (i.e. natural decomposition processes are reduced, ecosystem sustainability is threatened, or groundwater contamination is threatened), (ii) difficulties with contaminants of the waste material that may impede soil biological activity or may be resistant to mineralization (e.g. heavy metal contaminants and synthetic organic chemicals), and (iii) the potential for spreading of pathogenic microbes (e.g. fecal coliforms, protozoa, and viruses). For examples of how these potential difficulties are avoided, see review by He et al. (1992) regarding the land disposal of composted municipal solid waste. Societal benefit can result not only from the disposal of troublesome waste substances, but if the process is properly conducted, soil properties can also be improved (e.g. Douglas and Magdoff 1991; Epstein et al. 1976; Mays et al. 1973; Mitchell et al. 1978; Piccolo and Mbagwu 1990).

These limited examples of the impact of soil carbon cycle processes on the total terrestrial ecosystem emphasize the necessity to develop a clear understanding of the types of organic carbon compounds mineralized in soil, their distribution in major carbon cycle pools (Figure 10.1), and their decomposition kinetics.

10.2 Biochemical Aspects of the Soil Carbon Cycle

The complexity of the biochemical processes associated with carbon cycling in soil is exemplified by the diversity of organic compounds mineralized and the array of microbes involved. The soil microbial community is adept at converting native biomass components plus a variety of xenobiotic compounds to atmospheric carbon dioxide, albeit with varying rates dependent upon the substrate structure and ecosystem properties. This seemingly limitless capability of the soil microbial community to mineralize organic carbon compounds could be considered to be the ultimate in recycling. Indeed, at one time in the development of the concepts of soil microbiology, the soil microbial community was considered to be infallible. Failure to discern measurable mineralization of an organic compound was considered to relate to the inability of the researcher to provide conditions conducive to the activity of the requisite microbes, rather than to be an inherent genotypic limitation of the microbes. With the advent of the "xenobiotic chemical age," it has become apparent that the concept of microbial infallibility was at best optimistic from the view of microbial capacities and pessimistic in regard to the innovative abilities of chemists and the magnitude of the societal appetite for unique industrially synthesized materials.

As a result of the expanding capacity to synthesize organic carbon-based compounds of little resemblance to those produced naturally, this fallibility/infallibility concept of the soil microbial populations must be adjusted. More accurately, the soil microbial community can only be considered to be infallible at mineralizing biologically synthesized organic compounds. This conclusion is based on current concepts of genotypic diversity of indigenous soil microbes. Interestingly, the potential now exists to expand soil metabolic capabilities through bioengineering. With the increasing ability to develop "designer genes," the enzymatic array of the soil microbes may come to include a variety of "unnatural" enzymes. These would be characterized by the fact that they result from modifications of cellular DNA to allow the production of enzymes with a specific capacity to cleave molecular bonds not normally encountered in the soil system, thereby resulting in mineralization of the chemically synthesized organic compound. In that situation, perhaps, our concept of microbial infallibility may return to something approaching the optimistic view of the prechemical age. The challenge is for soil microbiologists to become as adept at developing novel degraders of xenobiotic compounds as chemists are at producing such substances.

10.2.1 Individual Components of Soil Organic Carbon Pools

The organic components of soil amendments and native soil organic matter are relatively easily separated conceptually by chemical class; that is, polysaccharides, proteins, lignin, humic acids, and fulvic acids. Each of these groups of compounds is distinguished by specific chemical properties that delimit its utility to serve as a carbon and energy source for the soil microbial community. Since all but the humic and fulvic acids are sufficiently defined chemically that they can be studied in the test tube, the biochemical processes associated with their mineralization appear to be reasonably predictable in soil. Thus a basic understanding of the biochemistry of the soil environment can be revealed through analysis of the organic compounds available as carbon and energy resources.

An understanding of the relative biodegradation susceptibility of each chemical group allows prediction of the relative activity of different soil ecosystems. For example, a site containing large quantities of polysaccharide would support a more active microbial community than one in which essentially all the organic carbon is associated with humic and fulvic acids. The former situation is exemplified by a soil system receiving significant inputs of fresh biomass (most productive, native ecosystems). Alternatively, the soil system characterized by a predominance of biodegradation-resistant humic substances would be one receiving little fixed carbon input from associated plant or animal activity. Thus, the indigenous microbial population would be expected to reduce the reserves of easily metabolized fixed carbon to a minimum. Then, the only remaining energy

source would be the more difficult to catabolize organic substances. Such a site is exemplified by a fallow (bare) soil maintained with little or no plant growth for several seasons.

Quantification of biologically decomposed substrates in a soil sample only provides at best an estimate of the potential biological activity contained therein. This conclusion results from the fact that the organic compounds do not exist in isolation in soil, but instead occur in complex mixtures of easily decomposed and more complex organic substances, not all of which are available to the soil microbial community. For example, proteins and starch are readily detected in soil. These substances may be decomposed within a matter of hours when amended as chemically pure preparations to soil, but both starch and proteins could have half-lives of days or longer in soil should they be retained within a reasonably biodegradation-resistant structure, such as a plant cell wall.

Polysaccharides are the primary energy source of the soil biological community. The predominant source of soil polysaccharides is plant tissue, although locally important contributions may be derived from influxes of animal biomass (e.g. amylose). Polysaccharides originating within the soil community are mainly the product of microbial synthesis. Between 5% and 25% of the carbon contained in native soil organic matter (i.e. that organic matter remaining in soil after all recognizable plant and animal debris has been removed) is composed of polysaccharides. Of this soil humus polysaccharide, that originating in newly synthesized biomass is most susceptible to biodegradation, whereas polysaccharides covalently bound to humic acids and those coating soil minerals are more resistant. A diverse mixture of polysaccharides and their decomposition products has been isolated from soil including:

- monosaccharides including hexoses (D-glucose, D-galactose, D-mannose) and pentoses (D-ribose, D-arabinose, D-xylose)
- polysaccharides such as cellulose and a variety of hemicelluloses from plants as well as amino sugar-containing polysaccharides, found in some fungi and in insect exoskeletons plus the complex mucopeptides of bacterial cell walls
- uronic acids and polyuronic acids such as glucuronic acid, galacturonic acid, and polygalacturonic acid (e.g. pectin)
- methylated sugars: 2-O-methyl-D-xylose, 2-O-methyl-D-arabinose, 2-O-methyl rhamnose, 4-O-methyl galactose.

For greater detail on this subject, see Stevenson (1994).

These polysaccharides are distinguished by the diversity of linkages between the monosaccharide units (cellulose vs amylose), inclusion of nonsugar moieties (chitin and microbial cell walls), as well as combination of a variety of monosaccharides within a single polysaccharide molecule (hemicelluloses). This variation in molecular properties impacts decomposition susceptibility through alteration

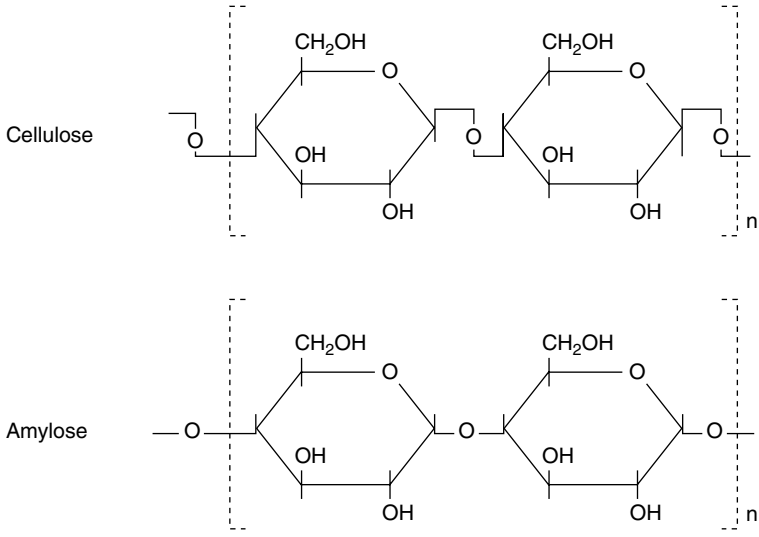

Figure 10.2 Structural formulae of cellulose and amylose.

of the crystalline structure of the molecule and/or the number of enzymes necessary for its mineralization.

The effect of linkage of the sugar moieties on biological stability is best exemplified by comparing the structures of two glucopyranose: amylose and cellulose (Figure 10.2). Amylose (α-(1→4)-D glucose) is usually decomposed in soil in a matter of hours whereas cellulose (β-(1→4)-D-glucose) has a half-life in soil that may be measured in years. Amylose is hydrolyzed to simple sugars by a single enzyme, amylase. In contrast, a complex of enzymes is required for disruption of the crystalline structure and cleavage of cellulose into monomers (e.g. Béquin 1990). Specific enzymes are required to disrupt the crystalline structure of cellulose before it can be hydrolyzed. Chitin (β-(1→4)-N-acetylglucosamine) also has a rigid structure that is resistant to most soil bacteria.

In contrast to the reasonably simple structures of the above polysaccharides, the bacterial cell wall (Figure 10.3) is composed of regular repeating units of N-acetylglucosamine and N-acetylmuramic acid interlinked by short peptide chains. A degree of biodegradation resistance is anticipated since these substances protect the easily metabolized substances comprising bacterial cytoplasm from degradation. Bacterial cell wall polymers are significant sources of mineral nitrogen following death and mineralization of the bacterial cell.

Hemicellulose is second in abundance in plant tissue only to cellulose. It is found in proximity to cellulose in primary and secondary cell walls. Structurally, the only commonalty between cellulose and hemicellulose is that they are both polysaccharides. Two groupings of hemicellulose are found in soil – homoglucans

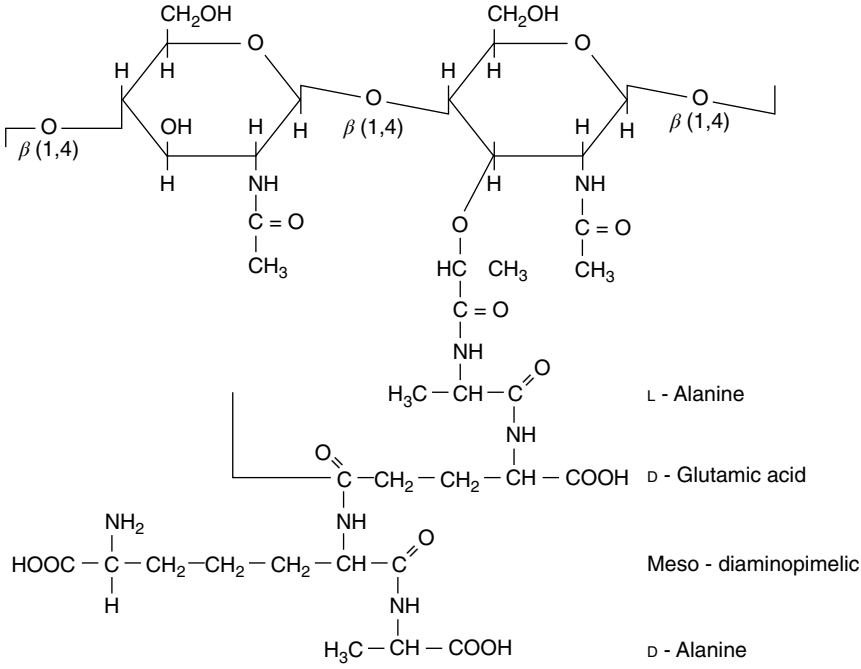

Figure 10.3 Structural formula for *Escherichia coli* peptidoglycan cell wall structure.

and heteroglucans. Homoglucans, the less common grouping, consist of single monosaccharide units. Examples include xylan (polyxylose), mannan (polymannose), and galactan (polygalactose). Heteroglycans are composed of more than one monosaccharide or uronic acid. Most contain 2–4 different monosaccharides, but examples of 5–6 components are detected. The most abundant moiety is listed last in the name, for example mannose for glucomannan and arabinomannan. Hemicelluloses are structurally complex, containing 50–60 monosaccharide units with branching. A small group of monosaccharides (xylose, arabinose, mannose, glucose, galactose, glucuronic acid, and galacturonic acid) provide the bulk of the sugar moieties of hemicelluloses.

Lipids are a more heterogeneous group of soil organic compounds. This soil component is defined operationally. Soil lipids are those soil organic components that are soluble in organic solvents. They include fats, waxes, and resins. Chemical components include waxes (long chain fatty acids and higher chain aliphatic alcohols and some cyclic alcohols), organic acids (both short and longer chain), normal paraffins (C_{16} to C_{32}) as well as polycyclic hydrocarbons. The latter groups include polycyclic aromatic hydrocarbons, sterols, terpenoids, and chlorophyl. (See Stevenson 1994 for a more complete discussion of these compounds.)

Lignin is a major component of plant biomass. Stems of woody angiosperms contain between 18% and 25% lignin (on a dry weight basis). For comparison, gymnosperms are composed of 25–35% lignin whereas 10–30% of the weight of monocotyledons is lignin (Crawford 1981). Lignin is a random polymer of sinapyl, coniferyl, and coumaryl alcohols containing a variety of complex organic linkages that are considerably less common than those in other plant substituents. Spruce (*Picea abies*), for example contains 48% arylglycerol-β-aryl ethers, 6–8% noncyclic benzyl aryl ethers, 9.5–11% biphenyl, 7% 1,2-diarylpropane structures, 9–12% phenylcoumaran structures, and 3.5–4% diphenyl ethers (Crawford 1981). Due to the complex aromatic structure of this plant component, lignin is decomposed slowly in soil (see Kirk and Farrell 1987 for discussion of the biochemistry of lignin decomposition). The most labile components of this complex molecule are the side chains and methoxyl groups. For example, after a six-month incubation period in a neutral sandy loam, about 23% of the ring carbons and 2-carbons and 39% of the methoxyl carbons of coniferyl alcohol units linked into model and cornstalk lignins were evolved as carbon dioxide (Martin and Haider 1979). (See Tate 1987 for a more detailed examination of the decomposition of this compound in soil.)

Summary points regarding the decomposition of lignin that are useful for evaluation of the impact of lignin on the rate of plant carbon cycling through soil are as follows.

- The complexity of the structure results in extended residence time for the plant carbon contained in lignin in soil.
- Although lignin contains a variety of chemical components that could yield energy to growing microbes, recovery of this energy by the soil microbial community is unlikely. The variety of components and their random assembly into the lignin molecule increase the quantities of energy that the microbes must expend to recover energy from the mineralization of lignin. This includes that energy expended in the production of enzymes involved in their mineralization.
- The extended residence time of lignin results in incorporation of significant portions of the lignin molecule into soil humic acids. This extends further the longevity of these carbons in the soil ecosystem.

Humic substances are the most biodegradation-resistant component of soil humus. Although lignin presents a formidable barrier to microbes instrumental in cycling organic carbon entering or contained within the soil ecosystem to mineral pools, an even more complex structure is encountered by soil microbial populations active in mineralization of humic and fulvic acids. Humic acids are random aggregates of aromatic and aliphatic moieties. Molecular weights for these substances have been shown to range from a few thousand to over 1 million daltons (e.g. Stevenson 1994). Malcolm (1990) specified a molecular weight range for soil

humic acids of 50 000–500 000 Da. Although traditional concepts of the generalized structure of humic acids are based on the assumption that these substances are predominantly aromatic, recent research has shown that humic substances are characterized by a range of aromaticities (Malcolm 1990). Some humic acids are predominantly aliphatic whereas others (as traditionally believed for all humic acids) are composed of nearly all aromatic ring-containing substituents. For example, Hatcher et al. (1981) calculated aromaticities for humic acids from a variety of climatic zones ranging from 35% to 92%. Thus the commonly encountered hypothetical structure of humic acids must be interpreted to contain representative aromatic and aliphatic moieties, but the ratio between these individual components differs from that shown in general models (see Stevenson 1994 for examples of these molecular models). See Chapter 1 as well as Piccolo (2001), Piccolo and Mbagwu (1990), and Piccolo et al. (2002) for further discussion regarding humic acid structures.

A variety of proteins and polysaccharides may be covalently bound to the humic acid molecules. Furthermore, organic compounds entering soil or produced therein may become associated with humic acids through a variety of associations (e.g. hydrogen bonding, van der Waals forces, ionic bonds). Individual components of the humic acid complex may be microbiologically synthesized aromatic compounds, xenobiotic compounds, such as pesticides, or even extracellular proteins and polysaccharides released into the environment by microbial and plant biomass either as a normal process of cellular metabolism or through death and decay of the biomass. Additionally, low molecular weight organic substances may become stabilized within the large humic acid molecule through noncovalent linkages (predominantly weak molecular interactions, such as hydrogen bonding and van der Waals interactions). These interactions may be nearly as stable as associations involving covalent bond formation (Dec and Bollag 1997). Also, stable, noncovalent interactions of humic acid and hydrophobic organic substances can occur in hydrophobic regions of the molecule (micelles or of smaller extent) of the humic acid molecule (von Wandruszka 1998).

The soil fulvic acid fraction contains a mixture of organic compounds that includes monosaccharides, oligosaccharides, polysaccharides, amino acids, peptides, organic acids, and fulvic acids. Removal of the defined organic compounds through ion exchange chromatographic procedures (Malcolm 1990) leaves the more ill-defined soil organic component, fulvic acid. Fulvic acid is composed of a light brown, low molecular weight organic material of only a postulated composition. These materials have a molecular weight range of about 1000–5000 Da (Malcolm 1990) and are more oxidized than humic acids. Their origin in soil is more problematic. They may be decomposition products of the larger, more complex humic acids, precursors of these molecules, or, more likely, both.

Humic and fulvic acids present a formidable, but not insurmountable, barrier to the microbes involved in mineralization of soil organic matter. A long list of papers have been published in which it is concluded that specific microbial strains with the capability of mineralizing these complex compounds have been isolated (e.g. Andreyuk and Gordienko 1978; Andriiuk et al. 1973; Blondeau 1989; Fedorov and Il'ina 1963). The fact that an equilibrium level of these substances is reached in soil further indicates that these substances are mineralized, albeit at a slow rate, in the soil environment. The latter conclusion is supported by the fact that ages of humic acids in soils have been estimated to range from a few decades to several thousands of years (Stout et al. 1981).

Examination of the structure of soil humic acids and of their distribution in soil reveals two sources of the resistance of these substances to microbial catabolism. Biochemical stability is derived in part from (i) the diversity of the components of the molecule and (ii) the random distribution of the individual components within the humic acid structure distribution. For a microbial species to utilize these complex molecules for a carbon and energy source, a large number of enzymes would have to be synthesized. The energy expended by the cell in producing the enzymes would most likely exceed that which could be derived from catabolism of the complex aromatic/aliphatic structure. Further impediments to microbial decomposition of humic acids develop from the association of the large molecule with soil minerals, especially clay particles. This chemical and physical interaction would inhibit approach of the molecule by either cells or enzymes (Theng et al. 1989).

Because of the diversity of the biological compounds associated with humic acids either through covalent linkage or a weaker association, the potential does exist for microbes to gain at least a limited amount of energy from catabolism of portions of the humic acid molecule. The most easily envisioned mechanism for energy production would be the mineralization of humified proteins and polysaccharides. Even the oxidation of these normally easily decomposed substances would be slow when they are associated with the large humic acid molecule. The approach of the protease or hydrolase would be inhibited physically by the obtrusive aliphatic/aromatic humic acid component. Thus, it can be proposed *a priori* that the organisms involved with mineralization of humified substances would be slow growing. This hypothesis appears to be true in that most reports of microbial growth on humic acids involve the activity of slow-growing bacteria such as *Actinomyces* spp., *Nocardia* spp. and related microbes (e.g. Andreyuk and Gordienko 1978; Andriiuk et al. 1973; Blondeau 1989; Fedorov and Il'ina 1963).

Linkage of the observations that (i) humic acid molecules are decomposed biologically, (ii) oxidation of the random structure of the aliphatic/aromatic portion of the molecule would not yield sufficient energy to support microbial growth, and (iii) humified polysaccharides and proteins could be oxidized to yield energy

suggests that in the vast majority of situations, the soil microbial community decomposes the aliphatic/aromatic humic acid core cometabolically. Energy for microbial growth could be supplied by oxidation of the humified proteins and polysaccharides as well as by nonhumified organic matter located in the same microsite as the humic acid molecule.

10.2.2 Analysis of Soil Organic Carbon Fractions

Difficulties in quantifying components of soil organic matter are not generally the result of shortage of analytical methods. An immense and diverse body of literature exists in which the quantification of xenobiotic chemicals or specific biologically synthesized compounds has been described. Primary difficulties result from attempts to apply the methods developed for analysis of reasonably homogeneous or mildly complex samples to chemically complex soil samples. Association of the target organic compound with humic substances or soil minerals reduces recovery efficiencies. Furthermore, the chemical and physical reactivity of soil organic components may be enhanced during the extraction period, thereby creating a diverse array of procedural artifacts. Thus, implementation of any analytical procedure for use with soil systems requires modification in order to maximize extraction efficiency and minimize artifact generation.

Analysis of a defined organic component (e.g. pesticides and other xenobiotics) in a heterogeneous matrix is complicated by the degree of interaction of various substituents with soil organic and mineral components. The intensity of the extraction procedure employed necessarily varies depending on the extent of association of the organic grouping of interest with the mineral and humic components, the complexity of the material to be extracted, and the solubility of the substance to be analyzed. The least complex procedure would be that applied to analysis of substances in soil interstitial water or easily separated from particulate substances; for example, the extraction of free carbohydrates, water-soluble proteins, or simple organic compounds (e.g. acetate, amino acids) with water or ethanol. As the solubility of the organic component in water decreases or the association with soil minerals increases, use of more stringent extractants is necessitated. Differential solubility is commonly utilized to separate soil mineral and organic fractions. This situation is exemplified by the utilization of ether or methylene chloride to extract aliphatic compounds. Perhaps the most extreme extraction procedure is the separation of humic substances from mineral matter with strongly alkaline solutions (usually 0.5 M NaOH).

A clear understanding of the limitations of each fraction procedure is necessary to determine its general applicability as well as to interpret the data yielded. Concerns range from the more mundane (e.g. how to remove the soil from the extraction suspension) to the highly critical determination of artifacts resulting

from the extraction procedure. Some specific questions regarding soil organic matter fractionation include the following.

- *What is the best method to reduce mineral contamination of the extract?* Soil extracts contain mixtures of the target compound plus dissolved soil minerals and suspended colloidal minerals, such as clays. The degree of contamination with soil mineral colloids and salts is termed the *ash content* of the extract. Since these substances interfere with many of the analytical procedures used to quantify soil organic fractions subsequent to extraction, they must be removed or minimized. Ionic contaminants may be removed with ion exchange resins. Mineral inclusions (e.g. clays) are reduced by pretreatment of the soil with hydrofluoric acid prior to extraction.
- *Does the extraction procedure modify the organic carbon pool?* Strongly acidic or alkaline solutions are frequently utilized to dissociate soil organic components from soil minerals. This may result in oxidation, hydrolysis, or even total destruction of some compounds. Polysaccharides and proteins may be hydrolyzed under acidic conditions. The oxidation state of humic acids is increased under alkaline conditions in the presence of oxygen. Furthermore, some amino acids are destroyed by heating in the acidic solutions generally used for extraction of proteins from soil.
- *Are the extractions quantitative?* Two questions are generally considered in evaluating a procedure for extracting organic matter from soil: Is the procedure efficient? Is the array of compounds contained in the extract representative of that existing *in situ*? An indication of the proportion of material extracted is required for determination of *in situ* concentrations but for many studies, a representative sample not total extraction is acceptable. As long as a representative, reproducible extraction of the organic matter is conducted, 100% efficiency is not required. Difficulties are encountered if not all components of a fraction are contained in an extract or if their ratio is altered by the extraction procedure. For example, should it be of interest to elucidate the diversity of polysaccharide monomers in an organic soil, all polysaccharide pools must be represented in the extract in proportion to their occurrence in soil. This mixture of sources includes the more easily extracted plant-associated materials as well as those less readily solubolized, bacterially synthesized heteropolysaccharides coating soil minerals.
- *How does the extraction procedure affect postextraction analysis?* Due to the complexity of soil and the intensity of the extraction procedure necessitated by the association of organic materials with soil mineral and humic components, it is not uncommon to produce a soil extract containing high concentrations of substances that interfere with subsequent characterization of extract components. Probably the best example of this situation is the fulvic

acid fraction. The high salt content in the fraction necessitates extensive purification of the extract before the individual organic components can be characterized.

Specific extraction procedures for analysis of soil organic matter typically involve dissolution of the organic matter in strongly alkaline or acid solutions (Stevenson 1994). All the questions listed above pertain to these procedures. These methods are characterized by the intensity of the extractants (highly acidic or alkaline solutions), incomplete removal of the fraction from soil, modification of the substance of interest during extraction, and production of an extraction solution that must be neutralized and desalted prior to analysis.

These limitations of extraction procedures create significant problems with data interpretation, particularly in comparing results attained with different soils or laboratories. Data must be interpreted with the understanding that extraction efficiencies may vary with soil type and that the degree of modification of the soil organic component during extraction may not be equivalent for different laboratories. The latter condition is particularly acute when examining historical data in that many early experiments were conducted without an appreciation of the potential for creation of artifacts by the extraction procedure. For example, the alkaline extraction of soils must be conducted under a nitrogen atmosphere to reduce oxidation of the organic matter. See Stevenson (1994) for a more detailed discussion of specific procedures used in isolation of soil organic matter fractions, their applicability, and limitations.

10.2.3 Structural versus Functional Analysis

As outlined in the previous section, procedures for analyzing organic components in soil are reasonably straightforward. For the soil microbiologist, the greater difficulty is derived from the fact that provision of a quantity and identity of a chemical entity in soil achieves only a minor portion of the objective. Experimental objectives usually require determination of a specific role of the organic materials in the soil ecosystem. None of the extraction procedures for soil organic matter used differentiate organic matter pools on a functional basis. More commonly, substances are extracted and quantified based on their solubility (e.g. humic substances), chemical class (e.g. protein, polysaccharide, lipid), or even distribution in specific biomass components (microbial, plant, or animal). Thus, the soil extracts contain a mixture of functionally active and inactive materials. For example, polysaccharides that are actively contributing to microbial respiration may be found in the fulvic acid, humic acid, and humin soil fractions. In contrast, protein, all of which can be quantified in an acid hydrolyzate of soil, is a mixture of protein directly contributing to soil respiration as well as biodegradation-resistant or

chemically stabilized protein (i.e. that protein stabilized by covalent linkage into soil humic substances).

The capacity to analyze soil organic matter fractions based on their role in the ecosystem is limited. One approximate division of soil organic substances of some functional value – at least conceptually – is separation based on decomposition susceptibility. Organic components may be classified as being easily (readily) decomposable or relatively biodegradation resistant. This elementary division allows discernment of that portion of fixed carbon that provides energy for growth and development of the rapidly dividing microbial populations and that fuels those more ephemeral processes commonly associated with hourly, daily or even seasonal changes in soil carbon as opposed to the more constant processes driven by the gradual decomposition of the more stable organic fractions.

By definition, readily metabolized soil organic matter provides a carbon and energy source for the soil microbial community with a minimal energy expenditure by the microbe. The microbial investment in energy acquisition involves synthesis of catabolic enzymes and direct substrate activation (as exemplified by substrate-level phosphorylation of glucose in the hexose monophosphate pathway of glucose catabolism). Compounds which require the action of a few enzymes prior to producing energy to the microbe are more easily decomposed than complex substances requiring interaction with a variety of enzymes prior to imposition of an oxidative change that provides energy to the microbe. The greater the energy deficit created by the microbe in preparation for catabolism of a substance, the less likely that the substance will serve as a primary energy source for the soil microbial community. For example, glucose and other monosaccharides; disaccharides, such as sucrose; amino acids; proteins; polypeptides; and simple aromatic compounds are all classified as easily metabolized compounds. Humic substances and lignin are extreme examples of more biodegradation-resistant substances.

In conclusion, a useful means of grouping compounds into readily decomposed and biodecomposition-resistant fractions is to evaluate the diversity of enzymes that must be synthesized to mineralize the substrate in relationship to the quantities of energy provided to the microbes. Simple organic compounds are easily decomposed and provide adequate energy to support viable microbial populations. Similarly, polysaccharides, although they may have extended longevity in soil as long as a few years, still yield growth-supporting energy with minimal expenditure by the microbes for enzyme synthesis. In contrast, considerable energy for microbial growth is contained in lignin and humic acids, but the energy expenditure involved in enzyme synthesis that would be required for the microbes to recover this energy in growth-supporting quantities is prohibitive.

Impact of physical distribution in soil on oxidation of readily metabolized carbon sources: Inherent properties of a chemical compound are primary contributers

defining its longevity in soil, provided that the substance exists in a free state and the microbial community (or their enzymes) enjoys unimpaired access to the material. Limitations to this access include the probability of interaction of the substance with the cell (i.e. the lower the concentration of the carbon/energy source, the less likely it is to be mineralized). Similarly, protection of the decomposable organic material by soil aggregates or inclusion within a partially decomposed or dead microbial cell reduces the substance's oxidation rate. Specifically in the latter situation, all cytoplasmic components, irrespective of their mineralization potential, are precluded from microbial attack until the plant or microbial cell wall is breached. Similarly, access to these energy-yielding substances is also precluded or limited sterically by linkage to soil humic acids.

Carbon dioxide yields from catabolism of easily metabolized substances: The role of readily decomposable organic compounds in soil carbon cycling is unquestioned. These compounds are the primary sources of energy and nutrients in an active soil ecosystem. Acetate catabolism in soil provides an excellent example of the decomposition kinetics and fate of this chemical grouping. After six days of incubation in soil, typically greater than 70% of acetate carbon is usually evolved as carbon dioxide, with the remainder incorporated into microbial biomass components, such as carbohydrates and amino acids (Sørensen and Paul 1971). Similarly, Kassim et al. (1982) found that as much as 90% of the carbon contained in readily decomposable compounds could be accounted for by carbon dioxide evolution Additionally, with inclusion of the carbon incorporated into microbial biomass into the calculation, a total of 95% of the readily decomposable carbon was accounted for.

These decomposition efficiencies are sufficiently reproducible that if they are not achieved where anticipated, the procedure must be carefully reevaluated. If less than about 70% of the carbon contained in a readily decomposable substance is evolved as carbon dioxide, either an error in experimental design may have occurred or some unanticipated soil property is inhibiting microbial activity. An example of the former situation is an incubation of a bulk soil sample where the soil moisture was not maintained throughout the experimental period. Declines of the soil water level to levels limiting microbial activity would result in an underestimation of the biodegradation potential of the soil microbial community. Alternatively, an environmentally meaningful result could have been occluded by a failure to account for all the potential fates of the compound. An experimenter may assume that all the amended substance should be mineralized to carbon dioxide. That reaction could occur, but insufficient carbon dioxide could be detected to account for total mineralization of the compound. This result could occur in a soil system where the pH of the soil favors conversion of carbon dioxide to bicarbonate and carbonate ($pH > 7$). The true quantity of carbon dioxide generated by carbon mineralization could be revealed by treatment of the soil sample

following the incubation period with hydrochloric acid, thereby allowing for the chemically retained carbon dioxide to be contained within the gas phase.

For comparison, a small portion of the carbon contained in a biodegradation-resistant compound is evolved as carbon dioxide, even after extended incubation periods. In a study of melanin decomposition, after a one-year incubation period, between 5% and 13% of the carbon had been evolved as carbon dioxide, with less than 0.7% of the carbon retained in microbial biomass (Stott et al. 1983). With this class of compounds, the bulk of the amended carbon is retained in unmodified or slightly changed parent molecules. The biodegradation-resistant compounds have an increased probability of being incorporated into soil humic substances (Tate 1987).

10.2.4 Microbial Mediators of Soil Carbon Cycle Processes

The concept of microbial infallibility presented above was soundly based on an appreciation of the diversity of microbial species that function as primary decomposers in soils. Biologically synthesized organic carbonaceous compounds are mineralized in essentially all soil systems where biological life is supported by the chemical and physical properties of the site. Organic carbon compounds are oxidized in soil with a variety of compounds, such as nitrogen oxides, molecular oxygen, organic substances, and sulfur compounds, serving as terminal electron acceptors. Active populations include primary decomposers (bacteria, actinomycetes, and fungi) as well as secondary feeders.

The breadth of organisms involved in carbon mineralization is exemplified by listing a portion of the microbial world involved in cellulose catabolism. This primary soil community energy source is used as a carbon and energy source by fungi (including *Alternaria*, *Aspergillus*, *Chaetomium*, *Coprinus*, *Penicillium*, *Phanerochaete*, *Polyporus*, *Rhizopus*, *Trichothecium*, and *Zygorhynchus*), yeasts (including *Kluyveromyces* and *Candida*), actinomycetes (including *Micromonospora*, *Microbispora*, *Nocardia*, *Streptomyces*, and *Streptosporangium*), as well as a wide variety of commonly occurring bacterial genera (including *Bacillus*, *Cellulomonas*, *Clostridium*, *Corynebacterium*, *Cytophaga*, *Pseudomonas*, *Sporocytophaga*, and *Vibrio*) (Béquin 1990).

Considering the vast number of microbial genera involved in carbon cycling processes, a functional division of cycle participants is commonly more useful in assessing metabolic potential and kinetics individual soil sites than is a detailed analysis of the microbial species present in the sample. Perhaps the most commonly encountered grouping of soil microbes is based on growth substrate and the rate of catabolism of the substrate. For example, soil bacteria may be grouped into such metabolic categories as amino acid oxidizers or cellulose degraders. The capacity of the carbon substrate to provide energy for growth and the capability of

the microbial species to compete with other members of the microbial community result in their separation into two groups based on growth rate. Rapidly growing soil bacteria, which are primarily involved in catabolism of easily metabolized or fresh biomass, are termed *zymogenous* bacteria. The slow-growing organisms (i.e. those predominantly associated with the steady decomposition of native soil organic matter) are classed as *autochthonous* (Winogradsky 1924).

Growth substrate/rate division of soil bacteria is useful to some degree but, as the metabolic diversity of the various species (especially members of the genera *Arthrobacter* and *Pseudomonas*) and their survival capacity in soil are elucidated, it becomes clear that an unequivocal separation of soil microbes into these defined groups is difficult. This delineation of soil bacteria is further complicated by the observation that growth rate of an organism is the result of the expression of genetically determined capabilities within the physical and chemical framework of the soil microsite. Thus, an organism that grows rapidly with an easily metabolized substrate as a source of carbon and energy source in the test tube may have generation times of days or longer in soil. Autochthonous or zymogenous designation becomes cloudy at best.

Other large-scale groupings that are commonly encountered, which are perhaps as tenuous as the autochthonous/zymogenous grouping, include division of the soil population into native and alien species and, a more modern apparition of this separation, natural vs bioengineered. As with the bipartite division of autochthonous and zymogenous bacteria, these designations are useful in clear-cut cases, but become confusing or even convey an erroneous impression of the diversity of the soil system with the more cosmopolitan or even with little-studied microbes. For example, organisms alien to the soil system could be cynically defined as those that have not to date been isolated from soil. In such situations, future studies would result in "alien" becoming "native." Further confusion may be derived from varying degrees of inclusiveness associated with native. Is a determination of native soil organisms related to a specific soil site, ecosystem, or all terrestrial soils? Difficulties with designations of bioengineered vs natural will likely emerge as nonengineered organisms with the same or nearly identical genotypes to the laboratory-produced microbes are isolated from soil.

A caveat associated with analysis of data in which soil microbes are divided into any of these general groupings is that the subjectivity of the decision process must be evaluated to assure that bias has not been introduced into the conclusions of the experimental study as a result of this artificial grouping of soil populations.

Many current studies have used phospholipid fatty acid analysis as well as DNA analyses to assess relationships of soil microbes due to plant growth as well as soil physical structure. Kravchenko et al. (2014) used 16sRNA pyrosequencing analysis to elucidate the influence of interaggregate pore structure on phylogenetic compositions of the bacterial community in macroaggregates. The results support

the conclusion that the legacy of the rhizosphere presence relating to interaggregate pore structure as well as microbial community composition can be sustained for 4–9 months. The data also suggested that sieving of soil samples could obscure meaningful changes in the microbial community composition.

Real-time quantitative polymerase chain reaction (PCR) is a method currently used to examine the impact of elevated carbon dioxide and moisture limitation on total bacterial 16S rRNA and denitrifier (*nos*Z genes). They found little impact of elevated carbon dioxide on bacterial abundance, as well as soil carbon and nitrogen concentrations. But in the microaggregates under low-moisture conditions, the microbial populations, as assessed via the PCR procedures, increased. That is, an impact of the low-moisture conditions was increased formation of microaggregates, which in turn supported enhanced microbial growth. Additional examples of such studies include those of Denef et al. (2009), who assessed impacts of management on microbial populations variation, and Ladygina and Hedlund (2010) who evaluated microbial population changes due to plant growth.

10.3 Kinetics of Soil Carbon Transformations

Identification and quantification of the proportion of the soil carbon distributed in various organic matter pools is both interesting and useful. But, in reality, from the view of determining the impact of this soil component on our ecosystem, movement of carbon between these various pools and rate of return to the atmosphere are better descriptors of the dynamics of carbon cycle processes occurring in any specific soil site. As discussed above, questions that are dependent upon carbon flux measurements (kinetics of the process) include evaluation of such terrestrially important processes as the capacity of the soil community to reduce or modulate atmospheric carbon dioxide concentrations and its utility in recycling organic wastes.

A variety of mathematical models have been developed in which carbon mineralization processes in soil and the effects of soil environmental properties are described (see Manzoni et al. 2014; Tate 1987). Based on the observation that most carbon mineralization results from biological decomposition, it would be reasonable to assume that the carbon transformations are best described by Michaelis–Menten kinetics (see Chapter 6). When evaluating the decomposition of simple biochemicals, their decomposition can easily be described by this simple descriptor of enzyme kinetics or similar equations describing microbial growth kinetics (the Monod equations). Unfortunately, because of the complexity of the populations catalyzing the processes, these models are rarely applicable in assessing microbial metabolic processes in soil.

More commonly, organic carbon-based substances decomposed in soil are a complex mixture of biochemicals that are decomposed by a variety of soil microbes as described above in a heterogeneous ecosystem. In this situation, carbon dioxide flux rates are a summation of the kinetics of individual biodegradation processes. Thus, any Michaelis–Menten type relationships could be obscured by the variety of active microbes present, the diversity of carbon-based substrates catabolized, differences in individual growth rates and enzyme efficiencies, and variation in the impact of the physical environment on the activity of the organisms and the enzymes. That is, kinetics of carbon dioxide evolution become the sum of all sources of this gas. Therefore, the rate of decay of these substances is frequently more easily described by a simple first-order equation with a series of terms for each biochemical group present in the complex substrate. A generic representation of the equation is as follows:

$$Y_t\left(\text{Biomass residue}\right) = C_1 e^{-k_1 t} + C_2 e^{-k_2 t} \ldots \tag{10.1}$$

where C_1 and C_2 are concentrations of the individual organic carbon fraction, t is time, and k_1 and k_2 are decomposition rate constants. An individual term could be added to this equation for each biochemical component of the complex organic substance studied, but experience demonstrates that the decomposition rates can be grouped into readily metabolized and more biodegradation-resistant fractions. That is, the decomposition of complex organic substances in soil is biphasic (Figure 10.4) with an initially rapid decomposition period (predominantly due to readily metabolized or labile organic carbon decomposition) followed by a slow decomposition phase (resistant and occluded organic matter). The magnitude of the rapid decomposition phase is proportional to the quantity of readily decomposed organic matter in the tissue and its accessibility. This phase could represent the majority of the carbon contained in the substrate (succulent green grass tissue mixed into soil) or a minority of the carbon (woody tissue).It must be noted that there is a certain amount of arbitrariness associated with the biodegradation susceptibilities associated with each portion of this biphasic curve. Some of the carbon dioxide produced during the rapid decline phase (initial decomposition portion of the curve) is the result of mineralization of a portion of the more resistant biomass components. These substances are clearly mineralized throughout the incubation period, just at a much slower rate than is associated with the easily decomposable pool. Furthermore, some of the carbon dioxide produced late in the incubation period is the product of mineralization of those easily metabolized compounds whose decomposition kinetics reflect those of the more resistant substances. As indicated above, this phenomenon is the result of the fact that the degradable substances are protected by the resistant materials (e.g. located within a plant cell wall) and thus can only be mineralized after the barrier is breached.

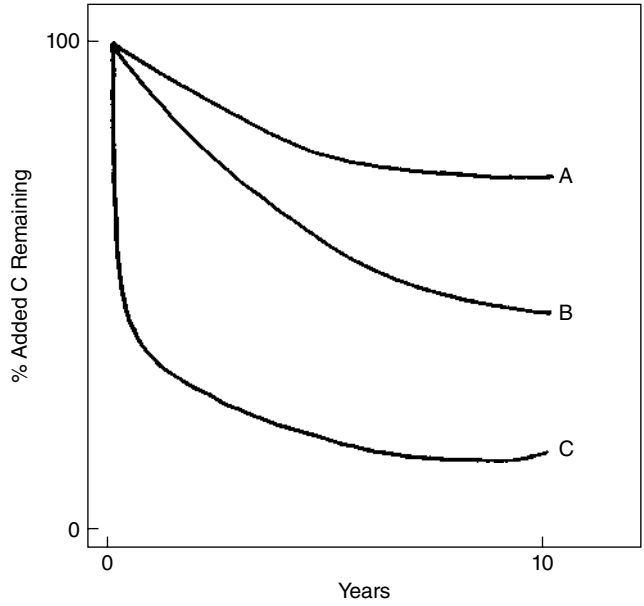

Figure 10.4 Biphasic decomposition curves for complex organic amendments to soil. The percent easily decomposable organic matter in A << B << C.

Decomposition kinetics of rice and barley straw (Murayama 1984), rye straw and maize (Jenkinson and Ayanaba 1977), and water hyacinth (*Eichornia crissipes* [Mart.] Solmes) (Moorhead et al. 1986) provide examples of the applicability of this two term equation in evaluating transformations of complex organic substances in soil. These materials are all constituted primarily of labile organic carbon. Therefore, most of their biomass is mineralized to carbon dioxide during the initial year following incorporation into soil. With these types of material, it is reasonable to anticipate that approximately two-thirds of the carbon will be evolved as carbon dioxide during the first year of incubation in soil. In contrast, first-year weight loss of leaf litter, which contains more biodegradation-resistant structures than do the succulent grass tissues and water hyacinths discussed above, has been shown to be considerably smaller (e.g. *Fagus grandifolia*, 21%; *Acer saccharum*, 32%; *Quercus alba*, 39%) (Shanks and Olson 1961).

These data were collected in aerobically incubated soil samples, but similar results can be derived from anaerobically incubated soil samples, if all decomposition products are considered. Gale and Gilmour (1988) derived rate constants for decomposition of alfalfa (*Medicago sativa* L.) in aerobically and anaerobically incubated soil samples. The aerobically incubated samples were incubated at optimum soil moisture under a carbon dioxide-free atmosphere whereas the anaerobic

soils were flooded under a nitrogen atmosphere. Carbon dioxide, methane, and water-soluble organic carbon were assayed. Three decomposition phases were distinguished (rapid, intermediate, and slow). Aerobic rate constants were 0.123, 0.059, and 0.0095 day^{-1} for each phase. The constants for the anaerobic decomposition were slower (0.118 and 0.024 day^{-1} for the rapid and intermediate phases, respectively). No constant was derived in this study for the slow phase.

These rate constants in the mathematical model are system dependent in that they are the result of the summation of all environmental parameters affecting the decomposition process in the individual site studied at the time of the experiment. Thus, the specific equation derived from any individual study can only be extrapolated to a system with similar properties, such as temperature, pH, moisture, and soil mineral content. The magnitude of the variation in these constants is not only shown in the above study with variation in oxygen tensions, but was also revealed in a comparison of the impact of climate on ryegrass decomposition kinetics in England and Nigeria. Decomposition of ryegrass and maize in Rothamsted, England, was described by the equation:

$$Y\left(\text{percent retention}\right) = 70.9e^{-2.83t} + 29.1e^{-0.087t} \tag{10.2}$$

where t represents time in years. The decomposition of these substances in Nigeria was described by the same equation, except that each decomposition coefficient was multiplied by four. Variations in the decomposition constants due to localized variation in seasonal soil moisture differences and soil type effects were also detected (Ayanaba and Jenkinson 1990). Should the experimental objectives include derivation of an ecosystem-independent mathematical representation of biomass decomposition in soil, then more extensive models than the two term model described herein must be derived (Tate 1987).

The data collected in the types of experiments described herein are commonly derived by assessing the evolution of ^{14}C-labeled carbon dioxide from soil samples amended with ^{14}C-labeled plant biomass. Thus, strictly speaking, in such studies the longevity of the carbon atom in soil organic fractions is being assessed rather than the survival of the actual plant biomass. This distinction is critical in conceptualizing the nature of the organic carbon from which the carbon dioxide is evolved in the latter stages of such a study. Initially, the vast majority of the carbon dioxide collected results from mineralization of added labile plant carbon. During this time of rapid plant biomass decomposition, a portion of the carbon mineralized is incorporated into soil microbial biomass. As time passes, some of the ^{14}C-label is incorporated into other soil organic matter fractions, such as humic substances, *in situ* synthesized polysaccharides, and stabilized proteins. Thus, during the slow phase of the decomposition curve (Figure 10.4), the ^{14}C evolved from the soil sample could originate in residual (more biodegradation-resistant)

plant components of the biomass initially added to the soil as well as from the mineralization of soil microbial biomass and humic substances synthesized *in situ* subsequent to amendment. For example, Sørensen (1987) found that after 8–15 years incubation in soil, the portion of the carbon originally added to a soil sample as barley straw was distributed in the soil amino acid fraction (21%) and microbial biomass (2.7%).

10.4 Conclusions: Management of the Soil Carbon Cycle

The basic properties of soil and the soil carbon cycle reviewed in this chapter demonstrate and support the conclusion that the soil microbial community is a basic mediator of a variety of soil processes required for development of a productive and sustainable ecosystem. Additionally, it is noted that appropriate management of our soils has both regional (e.g. agricultural and recreational uses) and worldwide implications (mediators of climate change). We can reasonably conclude that ecosystem productivity and sustainability rely to a large extent on the activity of the soil microbial community, especially for energy and nutrient transformations. Microbes are major players in sustaining the productivity of soils whether they are supporting food production, conducting those processes essential for sustaining our natural parks and other conservation sites, or assisting in completing the common cycles associated with disposal of organic wastes.

From the information provided in this text and examples of studies from the primary scientific literature, we conclude that in each of these roles, whether considering food productivity, management of organic wastes, or for esthetic enjoyment, the rate and direction of change of the organic carbon compounds are of greater importance than their mere presence. In soils at steady state, total soil organic carbon contents are relatively constant yet, even in these soils, large quantities of carbon may be cycling between various carbon pools. The products of this carbon cycling include the mineral and organic products required for sustenance of both aboveground animal and plant populations, as well as the soil biological community. These processes can be exemplified by comparing organic matter transformation in grassland soils compared to dry desert soils. In soils with relatively large inputs of fixed carbon, such as a grassland soil, enhanced inputs of carbon and energy result in a more active soil biological community, producing carbon dioxide and a variety of compounds shunted into soil organic matter. Not only will the biological activity increase but the participation of this community in soil improvement (e.g. aggregate production) could be enhanced.

On a global scale, soil site management has a major impact on world carbon balances, especially ecosystems in transition, as exemplified by intensively

managed agricultural soils. Such perturbation of soil properties leads to an altered equilibrium concentration of soil organic matter organic matter; that is, a new steady-state carbon content (and therefore, carbon dioxide flux rate) is achieved.

Soil organic matter contents may range from nearly zero (e.g. desert soils) to approaching 100% (e.g. the organic soils, Histosols, of South Florida). To a large degree, the equilibrium level of soil organic matter depends not only on inputs of organic matter but also on the basic properties of the soil itself and the impact of how the ecosystem is managed. Inappropriate management of a soil site, e.g. excessive cultivation, can result in reduction of soil aggregates and loss of soil structure that often leads to nearly total depletion of the native soil organic matter. In contrast, under swampy situations, soils (Histosols) consisting of essentially 100% organic carbon may be formed (i.e. the flooded conditions of the ecosystem result in preservation rather than loss of soil organic matter). With most mineral soils, a level of a few percent organic matter is usually attained and maintained, both in properly managed and native soil sites. The quantity of organic matter retained in a particular soil is the result of the achievement of a balance between inputs and mineralization as impacted by the physical and chemical properties of the site. Optimization of the organic content of the soil clearly leads to an optimization of soil productivity and sustainability.

Since organic matter synthesis and decomposition are primarily biologically controlled, they can be managed. Modulators of soil biological activity that may be anthropogenically managed include pH, temperature, nutrient concentration, and moisture. Therefore, in conclusion, an improved understanding of the basic transformations of soil organic matter and the environmental parameters controlling its fate allows for the development and application of the appropriate best management practices.

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11

The Nitrogen Cycle

Mineralization, Immobilization, and Nitrification

The nitrogen cycle consists of a series of oxidation and reduction transformations of nitrogen. The major processes occurring therein can be summarized as follows (Figure 11.1). Relatively inert atmospheric nitrogen (dinitrogen, N_2) is converted into a biologically available form (fixed nitrogen in the biomass of the microbe catalyzing the process). The reduction of dinitrogen into organic nitrogen is nitrogen fixation. This organic nitrogen is mineralized into ammonium (nitrogen mineralization) by decomposer populations. The oxidation of the ammonium to nitrate (nitrification) provides energy to the nitrifiers and produces a form of fixed nitrogen generally used by the plant community. Ammonium and nitrate may be assimilated into microbial biomass (immobilization). The most oxidized form of fixed nitrogen in soil, nitrate, is returned to the atmosphere as dinitrogen and nitrous oxide by organisms using the nitrogen as a terminal electron acceptor (denitrification).

For the practical objective of understanding the magnitude or relative importance of these processes in soil, the soil nitrogen cycle can easily be divided into two "minicycles": (i) a predominantly soil resident portion consisting of those processes (mineralization, ammonification, nitrification, immobilization) which are associated primarily with the conversion of organic nitrogenous compounds to plant-available mineral forms and their return to biomass (plant, microbial, and, indirectly, animal); and (ii) the more inclusive cycle (soil as well as the greater biosphere resident processes) entailing the conversion of atmospheric dinitrogen into forms available to the living components of the ecosystem (i.e. nitrogen fixation), the transformations within the soil described above, and the return of the fixed nitrogen to atmospheric dinitrogen (i.e. denitrification). The interrelationships of these two aspects of the nitrogen cycle are depicted in Figure 11.1.

The significance of this positional differentiation of the nitrogen cycle in native environments is readily exemplified and contrasted by comparison with the situation in a climax forest ecosystem and a newly developing soil system, such as

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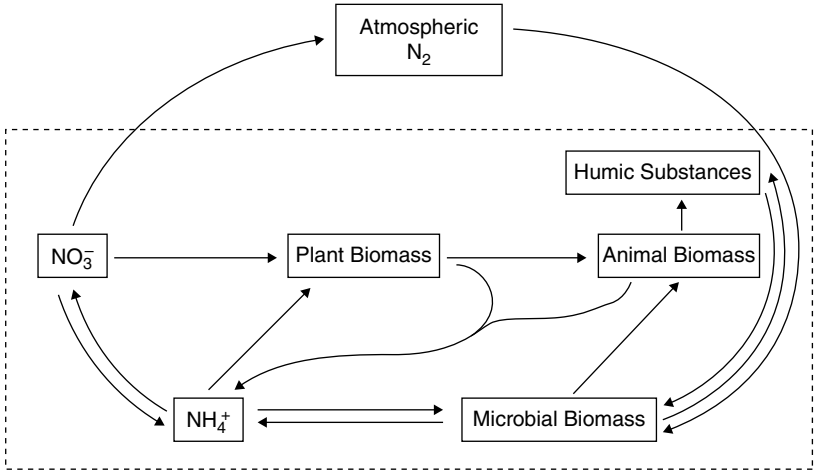

Figure 11.1 Conceptual model of the nitrogen cycle. Nitrogen reservoirs enclosed in the dashed lines are resident in soil whereas dinitrogen is replenished from atmospheric sources.

would occur with reclamation of a highly impacted mineland soil in which all naturally occurring organic matter has been destroyed. In the forest system, the use of fixed nitrogen sources from external sources (atmospheric deposition and nitrogen fixation) is minimal. As much as 85–90% of the fixed nitrogen incorporated into new plant biomass is derived from mineralization of soil humus and indigenous biomass. The approximately 10–15% of the fixed nitrogen lost from the system (via denitrification, leaching, runoff, etc.) is replaced by a low level of nitrogen fixation. In contrast, for a soil system with little accumulated organic matter, fixed nitrogen must be imported. This task is accomplished by nitrogen-fixing bacteria. For example, in a pioneer system (volcanic ash or even some mine spoils), indigenous fixed nitrogen resources are minimal. Plant biomass productivity is totally reliant on external resources. Nitrogen can be provided symbiotically by lichens or by free-living nitrogen-fixing bacteria (see Chapter 13) or through amendment with organic residues in managed systems.

In those situations where the bulk of the nitrogen utilized for plant biomass synthesis is the product of recycling soil resident nitrogen compounds between organic and mineral forms, homeostasis of the ecosystem can only be maintained if nutrient losses are minimized. Thus, it is advantageous for the majority of the fixed nitrogen contained in soil reservoirs to exist in nonmobile forms, such as organic or biomass nitrogenous compounds and, to some degree, ammonium ion. Fortunately, most of the fixed nitrogen in soil systems is retained in organic nitrogen pools. Typical data demonstrating the distribution of soil nitrogen in organic and mineral forms are presented in Table 11.1. Although soil nitrate and ammonium concentrations are

Table 11.1 Examples of nitrogen distribution in mineral and organic forms: agricultural soils from Valencia, Spain[a], and soils beneath shrub-steppe vegetation (south eastern Washington, USA)[b]

Soil	Organic N ($mg\ N\ kg^{-1}$ soil)	Mineral N ($mg\ N\ kg^{-1}$ soil)	Mineral N (%)
Sandy soils (Spain) (n = 9)	300–2470	0–86	0–7.1
Loamy soils (Spain) (n = 9)	710–2400	3–153	0.4–6.7
Clay soils (Spain) (n = 9)	720–3680	3–85	0.2–1.8
Shrub-steppe soils (n = 15)	1500–3300[c]	3.71–7.00	0.2–0.3

[a] From Serna and Pomares (1992).
[b] From Bolton et al. (1990).
[c] Total Nitrogen.

highly variable with time and position in the soil profile, note that these compounds at most comprise a few percent of the total soil nitrogen. In the examples cited, quantities of nitrogen in soil organic matter ranged from about 10-fold to 500-fold the mass of nitrogen in mineral forms. (For further discussion of nitrogen distribution in these various soil reservoirs, see Tate 1987 or Stevenson 1986 for a review of the topic, or Bolton et al. 1990, Cabrera and Kissel 1988a, Cassman and Munns 1980, Clarhom et al. 1981 or Serna and Pomares 1992 for examples of primary research into the nitrogen transformations involving these nutrient pools.)

This documentation of the predominance of organic nitrogen in soil ecosystems reveals a potential indicator of plant biomass production potential. Since plant biomass synthesis is frequently controlled by the availability of soil mineral nitrogen resources (other potential delimiters of plant productivity include soil moisture, phosphate or trace mineral levels, and a variety of soil physical and chemical properties), assessment of net nitrogen mineralization rates of the internal soil organic nitrogen pools and/or amendment rates of external fixed nitrogen resources can provide a valuable estimation of the fertility of an ecosystem. Nitrogen inputs from sources external to the soil biological community (fixation or soil amendment) are the primary determinants of ecosystem productivity in soils containing low levels of biologically decomposable organic matter or in cropped soils receiving inputs of industrially fixed nitrogen or organic residues.

A conceptual model of the dependence of plant community productivity and structure on nitrogen mineralization is depicted in Figure 11.2. Nitrogen mineralization provides a pivotal base for total biomass production. In an ecosystem with large reservoirs of organic matter (microbial, plant, and animal biomass plus colloidal soil organic matter), the quantity of fixed nitrogen available for plant biomass synthesis is controlled by the balance between nitrogen mineralization

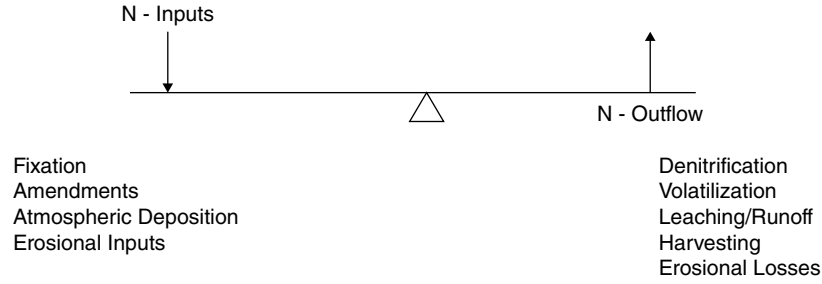

Figure 11.2 Processes contributing to soil nitrogen balance.

and immobilization ($N_{available} = N_{mineralized} - N_{immobilized}$). Due to the heterogeneity of soil and the fact that it is an open system, not all of this available nitrogen is retained in soil biomass. Some fixed nitrogen is lost through denitrification, volatilization, leaching, and runoff of soil water. The total quantity of fixed nitrogen within a soil system is also reduced by plant biomass removal (harvesting by animals in native sites and cropping in agricultural systems) and by soil erosion. (In reality, the fixed nitrogen in the latter reservoirs [harvested biomass and eroded soil] is not lost from the soil system in general, merely translocated to a different specific ecosystem.)

In a sustained ecosystem, nitrogen outputs must necessarily be balanced by inputs: nitrogen fixation, plant biomass inputs, atmospheric deposition, erosional deposition or anthropogenic intervention. In a steady-state system, a balance exists between the rates of mineral nitrogen generating/removal processes and plant growth. Modification of these rates results in establishment of a new biomass productivity steady state wherein these factors would again be balanced. Continued losses of fixed nitrogen without concurrent replacement would eventually result in creation of a biomass desert.

An appreciation of the pivotal role of nitrogen mineralization in the nature and productivity of the plant community of an ecosystem is essential in managed systems. Both excessive nutrient inputs and losses have negative consequences in regard to system sustainability. Fixed nitrogen decrements in agricultural systems commonly arise from crop removal, enhanced leaching of mineral nitrogen, and augmented soil erosion. Conversely, system overloads may occur through excessive organic matter amendments as may be encountered through disposal of sludge, composts, or animal waste materials or overuse of nitrogenous fertilizers.

Based on the essential influence of mineralization, immobilization, and nitrification on stability of soil ecosystems, the objective of this chapter is to evaluate the primary nitrogen cycling processes in soil and the properties of soil which control these reactions. Biological nitrogen fixation will be evaluated in Chapter 13 and denitrification in Chapter 14.

11.1 Nitrogen Mineralization

Simply stated, nitrogen mineralization is the conversion of organic nitrogen to inorganic nitrogen. Since, in soil, ammonium is usually rapidly oxidized to nitrate by nitrifiers, net nitrogen mineralization in an ecosystem is estimated by quantifying ammonium and nitrate concentrations. A determination of the absolute amount of nitrogen mineralized requires quantification of the nitrogen immobilized (see Chapter 10, Section 10.2). Since many studies involving assessment of nitrogen mineralization are concerned with determination of the quantity of fixed nitrogen available to the growing crop, most workers quantify net rather than total nitrogen mineralization. A more limited term, *ammonification*, is used to designate the conversion of organic nitrogen to ammonium. Mineralization is the sole, soil resident, biological means of generating the nitrogen forms usable by green plants.

11.1.1 Soil Organic Nitrogen Resources

Soil organic nitrogen is distributed between plant, animal, and microbial biomass, litter, and humic substance pools. Thus, determination of the biochemicals mineralized by the soil microbial community entails elucidation of the nitrogenous substances contained in biomass plus the more exotic humified substances. All nitrogenous compounds commonly contained in living cells are found in the soil organic fraction. Humified nitrogen, i.e. nitrogen linked to humic and fulvic acids, may be in recognizable biochemical forms (e.g. humified proteins and amino sugar-containing polysaccharides) or in heterocyclic ring compounds incorporated into the basic structure of humic or fulvic acids. (For more information on the chemistry of soil nitrogen associated with humic substances, see Stevenson 1994 for a review of the topic or Bondietti et al. 1972, Malcolm 1990, Piccolo et al. 1990 or Schnitzer and Kerndorff 1980 for examples of primary research.) It is interesting that Schnitzer and Kerndorff (1980) found that humic and fulvic acids from industrially and environmentally polluted soils contained more nitrogen and sulfur and less oxygen than did these substances isolated from unpolluted soils.

Those complex heterocyclic aromatic compounds contained in humic substances are of interest for developing a complete picture of the soil organic nitrogen component, but they contribute minimally to soil nitrogen cycling. Their rate of mineralization would be equivalent to that of humic acid in general. Nitrogen mineralization rates are determined essentially in totality by the quantity and availability of the more readily metabolized organic nitrogen compounds found in soil. These substances are composed primarily of amino acids and amino sugars and their oligomers and polymers. Other contributors include nucleic acid components plus, in managed systems, such societal products as various heterocyclic ring-containing pesticides. Again, except in the more limited situations where

these latter compounds enter the soil system in the high concentrations associated with accidental spills or mismanagement, they usually contribute minimally to overall soil nitrogen cycling.

This short list of classes of nitrogen-containing biochemicals creates an impression of simplicity of nitrogen mineralization processes that does not represent reality in soil. Biochemically, the process of converting organic to mineral nitrogen is the result of catalysis of a limited number of compounds by a few relatively commonly occurring enzymes (e.g. proteases and amidases). The predominant nitrogenous compounds (amino acids and amino sugars – monomers, dimers, oligomers, and polymers) are all relatively rapidly decomposed by a vast variety of soil microbes. Yet, it is reasonably easily demonstrated that nitrogen mineralization is not proportional to the quantities of these compounds present in the soil site, as could naively be predicted. For example, Fox and Piekielek (1978a,b) reported that after a two-year period, a variety of chemical soil nitrogen parameters correlated with nitrogen mineralization in soils from cornfields yet, with a longer testing period (four years) and greater variability in soil and weather, none of the tests were found to be accurate predictors of soil nitrogen mineralization (Fox and Piekielek 1983). Serna and Pomares (1992) found that prediction of soil nitrogen availability to plants in growth chamber studies required analysis of initial mineral nitrogen compounds of the soils plus several chemical and biological assays.

Similarly, Juma and Paul (1984) evaluated nitrogen mineralization dynamics using a ^{15}N-labeled Weirdale, loam soil. Their study of ^{15}N enrichment in mineral nitrogen products supported the conclusion that extraction of highly labeled organic N pools from the soil only partly explained the substrates used for nitrogen mineralization in the soil. Their data supported the conclusion that a variety of nitrogen pools interact in nitrogen mineralization processes. Note that here, a nitrogen pool or reservoir is *not* equated with or limited to a specific biochemical. For example, in this situation, nitrogen pools would include nitrogenous compounds in the relatively stable form of microbial biomass as well as the ephemeral pools of water-soluble amino acids, proteins, and amino sugars. Form or state of the biochemical is as important as the chemical identity in determining or designating soil nitrogen pools. Nitrogen-containing biochemicals may be bound to soil organic matter or particles, contained in plant, animal or microbial biomass or debris, or exist free in soil interstitial water or, simply, a biodecomposable organic nitrogen substance may be occluded within a soil aggregate. Each of these situations provides an unique impediment to access to the biochemical for liberation of the nitrogen contained therein. For example, humified compounds are protected by the inability of the enzyme to approach the substrate whereas microbial cells present a reasonably resistant cell wall barrier to protect internal biodegradable compounds.

Another aspect of the difficulty in using concentrations of decomposable organic nitrogen compounds as predictors of nitrogen mineralization potential is the fact that nitrogen mineralization may not be the primary reason for the conversion of the organic nitrogen to ammonium or nitrate. Organic nitrogenous compounds found in soil may also serve as sources of carbon and energy for the microbial community. Thus, any mineral nitrogen produced by these processes is a by-product of carbon and energy metabolism. For example, Learch et al. (1992) evaluated the mineralization of organic sewage sludge constituents in soils. The quantity of proteins extracted from the sludge was highly correlated with carbon mineralization but had limited relationship to the nitrogen mineralization rate. This observation supports the hypothesis that the soil microbial community was using the protein as a carbon rather than nitrogen source. In these situations, the carbon/nitrogen ratio tends to serve as a better indicator of nitrogen mineralization potential in that with a narrow ratio, a significant production of mineral nitrogen can be expected to occur whenever the microbial community uses the substrate for a carbon/energy source (e.g. DeLuca and Keeney 1993; Learch et al. 1992). (See discussion below regarding the interaction of the carbon/nitrogen ratio of the substrates metabolized by soil microorganisms and the resultant impact on mineral nitrogen production.)

11.1.2 Assessment of Nitrogen Mineralization

Soil nitrogen mineralization has traditionally been viewed from the viewpoint of its contribution to plant biomass production – generally in agricultural systems. It must be stressed that not all the nitrogen mineralized in soil is available to the plant community. Mineral nitrogen is removed from soil through assimilation by plants and microbes, it may be denitrified, and it may be leached from the root zone. Hence, change in soil mineral nitrogen could be represented as follows:

$$\Delta N_i = N_m - \left(N_a + N_p + N_v + N_l + N_d\right) \tag{11.1}$$

where N_i represents inorganic nitrogen, N_a is nitrogen assimilated by microbes, N_p is nitrogen assimilated by plants, N_l is nitrogen leached from the soil, N_v is nitrogen volatilized, and N_d is nitrogen denitrified. The number of terms in this relationship indicates the complexity of tracing the flux of nitrogen atoms between various soil nitrogen reservoirs in a field situation. The movement of this atom is simplified in laboratory-incubated soil samples.

Nitrogen mineralization potential, usually measured in laboratory-incubated soil samples, is most commonly estimated by assessing net mineral nitrogen production over a given time period in a closed system (batch soil samples or soil columns) in the absence of growing plants. Since such a system would be physically

isolated, leaching, volatilization, and denitrification losses are also minimized. Denitrification is generally reduced by maintaining aerobic soil conditions. Therefore, in these closed laboratory-managed, soil systems:

$$\text{Net}\,N_m = \Delta N_i = N_m - N_a \tag{11.2}$$

Thus, it is apparent that to understand the dynamics of mineral nitrogen production for plant biomass synthesis, the nuances of nitrogen immobilization must be evaluated.

11.2 Nitrogen Immobilization

11.2.1 Process Definition and Organisms Involved

Nitrogen immobilization is the microbial assimilation of mineral nitrogen compounds. Any actively growing microbe in soil contributes to this incorporation of soil ammonium and nitrate into microbial biomass. If the environmental conditions for microbial growth stall, nitrogen is immobilized. Thus, nitrogen immobilization occurs under such diverse environmental conditions as those associated with aerobic or anoxic soils, acid-impacted sites, and soils receiving hot spring or even nuclear power plant waste waters. The microbes that convert mineral nitrogen to microbial biomass are aerobes, facultative anaerobes, anaerobes, thermophiles, mesophiles, psychrophiles, acidophiles, neutrophiles, and alkalinophiles. That is, nitrogen immobilization is catalyzed by all groups of soil microorganisms.

Historically, the primary justification for study of nitrogen mineralization has related to its role in the loss of plant nutrient nitrogen from agricultural soils. In unmanaged ecosystems, studies of nitrogen immobilization have mainly involved evaluation of basic nitrogen cycling processes, such as the quantification of total nitrogen mineralization and the proportion of soil mineral nitrogen reservoirs cycled through microbial biomass. In contrast, in agricultural soils or other managed sites, such as reclamation projects, interest in nitrogen immobilization kinetics acquires an economic aspect since, quite frequently, the major justification of soil amendment with anthropogenically fixed nitrogen is to stimulate aboveground plant biomass synthesis. Excessive incorporation of the amended nitrogen into microbial biomass necessarily impacts plant community development.

11.2.2 Impact of Nitrogen Immobilization Processes on Plant Communities

The quantities of mineral nitrogen incorporated into the soil microbial population depend on community activity, which in turn in most aerobic soil systems is

controlled by the availability of carbon and energy resources. In anaerobic or oxygen-limited soils, moisture-limited sites, or soils of extreme acidity, microbial activity is usually sufficiently controlled by these factors that nitrogen immobilization is not a significant impediment to plant biomass productivity. In carbon-controlled communities, the impact of an influx of carbon-rich material on mineral nitrogen resources (i.e. ammonium and nitrate) depends upon the carbon/nitrogen ratio of the soil amendment. If the carbonaceous compounds providing carbon and energy to the active portion of the soil microbial community contain sufficient nitrogen to meet the needs of the soil microbes decomposing them, then a nitrogen deficient does not occur. Unfortunately, with most commonly utilized carbon and energy source in soil (plant biomass), this is not the situation. Plant biomass has carbon/nitrogen ratios as high as 60–80/1. Thus, since soil bacteria have carbon to nitrogen ratios of 4–6/1 and fungal ratios generally range from 10/1 to 12/1, synthesis of microbial biomass from these substrates necessarily results in a soil nitrogen deficit. That is, some soil nitrogen must be incorporated into microbial biomass to balance the carbon inputs. In soils where insufficient mineral nitrogen exists to compensate for the deficiencies of the carbon and energy source, the microbial growth rate is reduced by the nitrogen limitation. In either situation, a reduction in total soil mineral nitrogen resources by immobilization results in a loss of plant biomass productivity.

The differential fate of carbon and nitrogen contained in the organic matter mineralized by the soil microbial community is reflected in the change in the carbon/nitrogen ratio of the organic matter as it is transformed from plant biomass into soil organic matter. With the decomposition of biomass, the carbon contained therein is oxidized primarily to carbon dioxide with a small portion being incorporated into microbial biomass components. The nitrogen in the substance being decomposed is conserved in the soil site. Thus, the effective carbon/nitrogen ratio of soil humus decreases with time. Field data indicate that after the carbon/nitrogen ratio of the biomass residues amended to soil reaches about 30/1, net nitrogen immobilization is no longer a problem. At this point, instead of consuming mineral nitrogen, the catabolism of the plant biomass becomes a mineral nitrogen source; that is, there is net nitrogen mineralization within the system.

If the microbes decomposing the plant residue have an average carbon/nitrogen ratio of 10/1, why is nitrogen not limiting until the metabolized substrate carbon/nitrogen ratio approaches 10/1 rather than 30/1? At least a partial answer to this question is derived from analysis of the basic biochemical composition of the plant biomass. It must be remembered that a portion of the plant biomass entering the soil system is of limited metabolic availability to the soil microbes. These carbonaceous substances include lignin and lignified substances, easily metabolized substances protected by the more slowly decomposed plant cell walls, plus substances which become associated with and thereby become protected by soil

mineral matter such as soil clays. Lignin has a high carbon to nitrogen ratio and is a major component of plant biomass. Thus, the effective C/N ratio of the substance actively metabolized by the soil microbial community may be much lower than the overall ratio detected by gross analysis of the decomposing organic matter. That is, the portion of the plant biomass mineralized by the soil microbial community is essentially the total plant biomass minus the high-carbon, low-nitrogen lignin component.

The rate of decomposition of even the more readily decomposed organic substances providing carbon and energy to the soil microbial community affects the impact of the amendment on nitrogen immobilization. This fact is exemplified by two studies, one using relatively simple carbon compounds, the other with complex plant components. Kelley and Stevenson (1987) added glucose, a phenolic glycoside, or catechol with ^{15}N-labeled ammonium sulfate to soil and evaluated nitrogen immobilization. Most rapid immobilization occurred in glucose-amended soils (maximum incorporation at six days) compared to maximum incorporation times of 10 days with the phenolic glycoside and 20 days with catechol. It should be also noted that in their study, Kelly and Stevenson found that at maximum immobilization, the nitrogen incorporated into microbial biomass was 3.5–6-fold more susceptible to mineralization than was native soil nitrogen. Thus, more recently synthesized, most likely less humified, organic nitrogen compounds are more metabolically available to the soil microbial community. Similarly, Mengel and Schmeer (1985) evaluated the effect of straw, cellulose, or lignin soil amendments on nitrogen immobilization with the overall objective being to increase the retention of nitrogen in soil as organic nitrogen, thereby reducing denitrification losses. These workers found that straw and cellulose stimulated greater nitrogen immobilization than did lignin in the 36-day incubation period. This observation relates to the biodegradation susceptibility of the substrates in that little microbial biomass synthesis resulted from amendment of the soil with lignin due to its inherent resistance to microbial catabolism. The complexity of the soil nitrogen cycle interactions was demonstrated in that the study found that straw and cellulose amendment also stimulated the denitrification rate in amended soils compared to uncropped soil. Thus, incorporation of added nitrogen into the crop was higher in the unamended soils than in those receiving exogenous carbon sources.

These observations suggest that the percent nitrogen of organic material entering soil declines or increases until it approaches the percent nitrogen content of the soil microbial biomass. In reality, this change can be predicted and is observed in soil systems receiving large influxes of organic material, but the final carbon/nitrogen ratio achieved in a soil system at equilibrium is considerably greater than that detected in axenic cultures of bacteria and fungi. This limitation results from the fact that in a biologically active mineral or organic soil, there is a significant

concentration of soil humic substances (humic and fulvic acids plus humin). These humic substances have very wide (60–80/1) carbon to nitrogen ratios. Recall that these soil organic components are only minimally susceptible to catabolism by the biological community. The presence of these substances obscures changes induced by the amendment of soil with plant biomass.

The carbon/nitrogen ratio of native soil organic matter is a mean of the average 10/1 ratio of the microbial community and the elevated value of the soil humic substances. For example, Post et al. (1985) measured the C/N ratio of the humus from a variety of soil ecosystems. The ecosystems were from sites with varying climate and aboveground vegetation communities. They found that all but three samples had C/N ratios ranging from 10.2 to 30.2 with a mean of 16.0 for all soil types studied and a mean of 17.8 when the values were weighted for the variable number of samples per ecosystem type studied. The exceptions were a subtropical wet forest (C/N ratio = 3.3) and two soil samples from a tropical thorn wood land (C/N ratio = 9.2). Wet and dry ecosystems from a variety of climates were evaluated. Although none of these mean values for the C/N ratios of soils can be considered to be representative of all soils, they do provide an indication of the situation in steady-state native soil ecosystems.

11.2.3 Measurement of Soil Nitrogen Immobilization Rates

Measurement of total nitrogen mineralized requires quantification of the soil nitrogen immobilization rate. This assessment generally requires labeling of nitrogen atoms so that their movement between various soil nitrogen reservoirs can be traced. This task is accomplished through the use of ^{15}N. Mineral nitrogen compounds enriched in ^{15}N may be added to soil samples and the incorporation of the labeled nitrogen atom into organic nitrogen pools can be quantified. Nitrogen mineralization can similarly be assessed by quantifying the enrichment of ^{15}N in mineral nitrogen pools subsequent to soil amendment with ^{15}N-enriched organic matter. Since nitrogen already occurs in all of the soil nitrogen pools, the value measured in these experiments is the rate of enrichment of the existing pool with the ^{15}N. Limitations of this procedure, aside from availability of the mass spectrometer necessary to detect the ^{15}N, are involved with mixing of the labeled substances into soil. Calculations of nitrogen mineralized or immobilized by this technique are based on the assumption that a distinct nitrogen pool is not created by the soil amendment. That is, the added ^{15}N-labeled substance is of the same availability to the soil microbial community as are the indigenous nitrogen reservoirs. Due to the heterogeneity of soil, this assumption is likely rarely met, but this procedure yields data which are a reasonable estimate of the rate of *in situ* processes.

There has been some interest in developing soil extraction procedures which are specific for recently immobilized nitrogen compounds. For example, He et al. (1988)

and Kelley and Stevenson (1985) demonstrated that milder extraction procedures (e.g. hot water, hot 10 mM $CaCl_2$) removed more of the recently immobilized nitrogen than did the more intensive procedures (e.g. acidified permanganate). Although some enrichment of immobilized nitrogen was achieved with these procedures, the nitrogen contained in the extracts was still derived from a variety of soil pools. Serna and Pomares (1992) evaluated a combination of biological methods (plant uptake and aerobic soil incubations) and chemical extraction procedures (autoclave, 0.5 M $KmnO_4$, 6M HCl, or 0.01 M $NaHCO_3$) as predictors of nitrogen availability to maize in calcareous soils. They found no extraction method was an effective indicator of the concentrations of organic nitrogen compounds which can be metabolized by the soil microbial community, but predictive capabilities were improved by combining results from assessment of initial mineral nitrogen contents of the soils plus several chemical and biological assays in regression analyses.

These observations confirm that nitrogen immobilization is an essential function in soil microbial communities in that it represents the proportion of nutrients incorporated into the microbial community. From the view of the total ecosystem, the nitrogen immobilization pathway provides an alternate fate (e.g. incorporation into plant biomass vs soil microbial biomass) of mineral nitrogen. This concept provides a contrast to the viewpoint of the agriculturist and, to some the degree, an ecologist or reclamation specialist. To the former, optimizing aboveground crop production is of primary importance, whereas the latter may be more concerned with ecosystem sustainability. For those optimizing or maximizing biomass production, immobilization represents a troublesome economic problem. With each nitrogen atom incorporated into the microbial biomass, additional external nitrogen inputs are necessitated to maximize achievement of the desired end of an attractive and/or productive aboveground community development.

11.3 Quantitative Description of Nitrogen Mineralization Kinetics

Net nitrogen mineralization in native soil systems is generally observed to follow first-order kinetics (see Section 9.5.4). Its kinetics are approximated by the equation

$$dN/dt = -kN \tag{11.3}$$

where N is the concentration of mineralizable nitrogen, k is a rate constant, and t is time. Thus, an integrated form of this equation commonly used to calculate net nitrogen mineralization is as follows:

$$N_m = N_O\left(1 - e^{-kt}\right) \tag{11.4}$$

where N_m represents the net nitrogen mineralized, N_O is the organic nitrogen at zero time, and k and t are a rate constant and time, respectively. It must be noted that situations may also exist where zero-order net nitrogen mineralization kinetics are detected. Nitrogen mineralization is independent of the organic nitrogen concentration. In this situation, nitrogen mineralization kinetics are described by the equation:

$$dN / dt = k \tag{11.5}$$

where k is a rate constant. Occurrence of zero-order kinetics can be predicted to occur in soils amended with high concentrations of nitrogen-rich organic substances (e.g. bacterial or fungal biomass waste substances from fermentative, industrial processes). In this situation, the capacities of the mineralizer population are saturated. Increased mineralization cannot occur without an increase in the enzymes or population densities of the organisms catalyzing the process.

The alternative mathematical model most commonly used to describe nitrogen mineralization in soil samples is the double exponential model. As indicated above, organic nitrogen occurs in a variety of forms and physical situations in native soil samples. Some organic nitrogen compounds are easily decomposed by the microbial community whereas others are inherently biochemically resistant or physically occluded. In reality, each class or specific organic nitrogenous compound has a characteristic decay rate, but field data indicate that these substances can reasonably easily be grouped into two categories: those relatively rapidly metabolized and a more resistant reservoir. Thus, the above model can be expanded to include both organic nitrogen reservoirs as follows:

$$N_t = N_O S\left(1 - e^{-ht}\right) + N_O\left(1 - S\right)\left(1 - e^{-kt}\right) \tag{11.6}$$

where N_t is the nitrogen mineralized in time t, N_O is the potentially mineralizable nitrogen, S and (1 – S) represent the labile and less accessible organic nitrogen fractions of the total organic nitrogen pool, and h and k are decomposition rate constants for the labile and less accessible pools, respectively. Deans et al. (1986) applied this double exponential model to a variety of historical studies and found that it provided a good description of the net nitrogen mineralization kinetics. Other mathematical representations of nitrogen mineralization include empirical models (e.g. Broadbent 1986) and a variety of double exponential and mixed order representations (e.g. Bonde and Rosswall 1987; Bonde and Lindberg 1988). A caveat in deriving nitrogen mineralization constants from application of these mathematical expressions is that the values derived may vary depending on the methods used to fit the data to the model (e.g. Ellert and Bettany 1988; Juma et al. 1984; Talpaz et al. 1981).

Extrapolation of laboratory-derived net nitrogen mineralization rates to field situations: Independent of which mathematical model is used to analyze nitrogen mineralization data, application of the laboratory-derived results to field situations is dependent upon a number of assumptions. The primary assumptions that must be considered are (i) soil organic nitrogen mineralization occurs in the laboratory-incubated samples at rates comparable to those existing in the field, (ii) preparation of soil samples for laboratory analysis does not alter the availability of various soil organic nitrogen reservoirs to the microbial community, and (iii) many workers assume that nitrogen mineralization is a first-order process; that is, alternative models are not evaluated in the data analysis process. In reality, all of these assumptions are of questionable validity, at best.

The difficulties with the assumption that laboratory nitrogen mineralization kinetics reflect field rates are readily exposed when it is considered that nitrogen mineralization kinetics vary widely with temperature and moisture (e.g. Addiscott 1983; Cabrera and Kissel 1988b; Cassman and Munns 1980; Kladivko and Keeney 1987; Myers et al. 1982; Smith et al. 1977; Stanford et al. 1975) whereas in the most commonly implemented laboratory incubation methods, these soil ecosystem properties are controlled within a limited range. Cassman and Munns (1980) derived mathematical models to compensate for field variation in temperature (15, 20, 25, and 30°C) and moisture (0.1, 0.3, 0.7, 2, 4, and 10 bars). Marion and Black (1987) used empirically derived models to include the effect of time and temperature on nitrogen mineralization kinetics in Arctic tundra soils. Temperature variability may be compensated through use of the buried bag procedure (Poovarodom et al. 1988; Westermann and Crothers 1980), but field moisture variation is limited within the bag. Utilization of empirically derived relationships or even widely applied models, such as Q_{10} or the Arrhenius relationship, is useful in adapting laboratory parameters to the more variable field situation.

The second assumption, that collection and preparation of soils for laboratory incubation has minimal effect on nitrogen mineralization kinetics, is invalidated by a consideration of the soil properties controlling the availability of the various soil organic nitrogen pools to the microbial community. It has already been stated that a primary controller of nitrogen mineralization is accessibility of the substrates to the microbes and enzymes. Physical handling of soils as occurs during collection and sieving of samples clearly alters the occlusion of organic compounds within soil aggregates and thereby their susceptibility to biological decomposition. For studies of the impact of this liberation of soil organic matter in sample preparation on kinetics and the development of mathematical models to account for this laboratory-generated artifact, see Beauchamp et al. (1986), Crasswell and Waring (1972a, b), Cabrera (1983), and Cabrera and Kissel (1988a, b). Since it is appreciated that even the minimal handling of soil samples associated with the buried bag procedure results in alteration of nitrogen availability and

thus the nitrogen mineralization parameters derived from the study, it can be concluded that currently no method exists to negate the impact of sample handling on estimation of soil nitrogen mineralization potential.

A final consideration in estimating field nitrogen mineralization parameters is their spatial and temporal variation. Among the many factors limiting laboratory analysis of this soil community property is the reality that only a limited number of soil samples can be analyzed and that the incubations require several weeks for completion. Insufficient numbers of soil samples are generally assayed to overcome the impact of variability of nitrogen mineralization rates in the field in space and time. Nitrogen mineralization is highly variable across the landscape and changes significantly on a microscale with variation of limiting physical and chemical parameters. Goovaerts and Chiang (1993) found that microscale nitrogen mineralization variability (<1 m) was explained to a large degree by the distribution of soil oxidizable carbon which correlated with gravimetric water content. This microscale variation impacts the nature of a representative soil sample. Goovaerts and Chiang estimated that 24–37 samples were needed to estimated nitrogen mineralization potential (with a precision of 0.1 at $p = 0.05$) for a 1600 m^2 plot. The impact of variation with time requires incorporation of data describing variability of primary soil properties controlling nitrogen mineralization into the analysis procedure. Similar results were presented by Starr et al. (1992) in determining soil sample size and number requirements for assessing field variation of soil nitrate concentrations.

The basis for this spatial variation in nitrogen mineralization potential relates to properties of the macro-soil ecosystem and the micro-structure of the system. The former is exemplified by studies of the impact of plant distribution on soil organic nitrogenous compounds and nitrogen mineralization potential (e.g. Bolton et al. 1990). Typically in such studies, soil organic nitrogen as well as associated biological activities are concentrated within the portions of the soil profile that are under direct influence of the growing plant; that is, the rhizosphere and areas impacted by plant litter. The rhizosphere effect may be direct through the addition of plant-derived organic matter to the soil system or indirect as a result of the improvement of soil properties conducive to microbial population development. The latter may include moisture preservation due to elevated soil organic matter levels, temperature modulation on hot summer days under plant canopies, as well as improved soil structure due to encouragement of granule formation by plant roots.

Variation in the distribution of soil organic nitrogen reservoirs is also associated with soil structural development. Separation of soil fractions by particle density or size results in fractioning of soil organic matter into more readily decomposed and more biodegradation-resistant portions. Mineralizable carbon and nitrogen tend to be associated with smaller particle sizes (Anderson et al. 1981; Cameron and Posner 1979; Schnitzer and Kodama 1992). These fractions tend to have a lower

carbon to nitrogen ratio, with most of the mineralizable soil nitrogen found in the fractions with smaller diameter particles. Similarly, separation of soil based on density fractionation results in enrichment of readily decomposable organic matter (hence, an enrichment for higher nitrogen mineralization potential) in light fractions (i.e. specific gravity greater than $1.2\,g\,cm^{-3}$) (Sollins et al. 1984).

11.4 Microbiology of Mineralization

Nitrogen mineralization is essential for development of a sustainable ecosystem. Hence, it is reasonable to conclude that organic nitrogenous compounds are converted to mineral nitrogen in any situation where life is possible. Were this not a basic truth, the biotic community would require a continued input of mineral nitrogen and would have to adapt continuously to increasing levels of accumulated organic matter. This situation is rare, if it ever occurs, in natural soil systems. Plant biomass accumulates in swampy ecosystems, but aboveground plant production is balanced with the slow nitrogen mineralization rate occurring in waterlogged soils associated with the swampy conditions. Situations in which soils receive constant inputs of mineral nitrogen are, in our society, either fertilized agricultural or landscaped situations (wherein the fertilization regime presumably balances plant production needs) or pollution-impacted situations (such as soils receiving nitrogen-laden industrial effluents).

Thus, based on occurrence of nitrogen mineralization in essentially any ecosystem where life can occur, it is predictable that an equally diverse microbial community is capable of catalyzing the process. Nitrogen is mineralized by a diverse group of bacteria, fungi, and actinomycetes. These organisms are aerobes, anaerobes, thermophiles, mesophiles, psychrophiles, as well as metabolically diverse in regard to their energy needs. Specific microbial species active in this process represent all microbial genera growing and reproducing in soil. Indeed, quantification of nitrogen-mineralizing populations in soil is limited not by the number present in the soil sample, but rather by the ingenuity of the individual devising the growth medium for the populations. Common nitrogen substrates used to isolate organisms involved in nitrogen mineralization include protein, chitin, and amino sugars.

11.5 Environmental Influences on Nitrogen Mineralization

In that the processes associated with nitrogen mineralization and immobilization are basic to microbial life in general, it is reasonable to assume *a priori* that these biological catalyzed portions of the nitrogen cycle occur under essentially any

environmental condition wherein microbial growth is possible. Simply stated, nitrogen immobilization is the incorporation of mineral nitrogen into cell biochemicals. Thus, nitrogen immobilization is easily concluded to be a major by-product of microbial growth. In contrast, the direct association of nitrogen mineralization with carbon mineralization provides an indirect link between the former process and biological energy generation. Thus, the questions in regard to environmental influences on nitrogen mineralization and immobilization relate not to whether the processes occur but rather to the impact of modification of soil physical and chemical properties on the rate of these nitrogen conversions.

Primary ecosystem properties that are usually evaluated for their impact on nitrogen immobilization and mineralization rates are nitrogen concentration, soil moisture, pH, and temperature. Nitrogen concentration effects were considered above with the discussion of net nitrogen mineralization rates. The impact of moisture, temperature, and pH variation on these processes follows a pattern similar to that which has been observed for most biologically catalyzed processes (Figure 11.3). There is a minimum and maximum level of the stress factor wherein no activity is possible. Between these limiting values, the biological activity tends to increase to a maximum level at the optimal adaptation point of the stress factor, after which the biological activity again declines. This generalized reaction of the

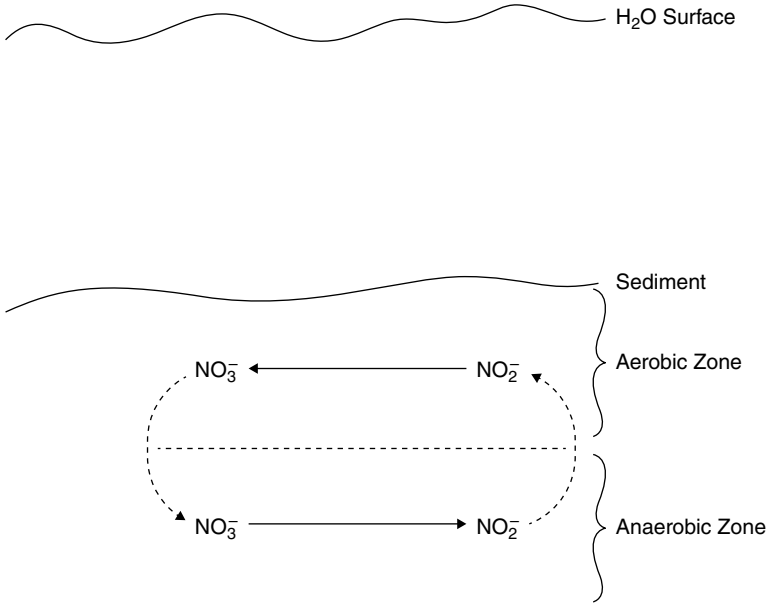

Figure 11.3 Nitrate-nitrite cycle involving diffusion of nitrate and nitrite between aerobic and anaerobic zones in sediments.

microbial community is exemplified best by analyzing the impact of soil moisture on nitrogen mineralization. Again, a generalized description of the process is presented, since individual reaction rates at specific soil moisture levels vary with the nature of the microbial community existent in a particular soil ecosystem.

With no other limitations, microbial growth, and thus nitrogen mineralization and immobilization, tends to increase as water availability increases. An optimal level of activity is generally reached at approximately water saturation. At this water level, adequate soil water is available for the microbes to interact with their growth substrates and for by-products of their metabolism to be removed from the cells' environs. As the soils become flooded, oxygen diffusion becomes a limiting factor to microbial respiration. Once the oxygen tension is reduced by an imbalance between consumption and influx of free oxygen, cellular processes become limited. Nitrogen mineralization and immobilization rates are reduced as the activity of soil microbes in general declines due to the anoxic conditions. Both nitrogen mineralization and immobilization do continue under anaerobic conditions, albeit at a generally reduced rate compared to that occurring under oxygenated conditions. Optimal soil moisture conditions for aerobic transformations of nitrogen generally range between 0.3 bar and saturation (60–90% water-filled pore space) (e.g. Walters et al. 1992), whereas the optima for pH and temperature are near neutrality and within the thermophilic range (40–60°C), respectively. Clearly, although most soils are not thermophilic and major active ecosystems have soils with pH values substantially below neutrality (e.g. tropical and temperate forest soils), these processes still occur at rates sufficient to sustain highly productive ecosystems under the less than optimal conditions of most soils.

11.6 Nitrification

Nitrification is the biological formation of nitrate and nitrite from compounds containing reduced nitrogen. These oxidative reactions are catalyzed by two, mutually exclusive, groups of organisms: ammonium and nitrite oxidizers. The primary reactions involved are as follows:

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$$

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

These processes involve the direct incorporation of molecular oxygen into the nitrogen oxide products. Note that hydrogen ions are produced by ammonium oxidation. Conversion of ammonium to nitrate results in a change in the oxidation state of the nitrogen from its most reduced state (−3) to the most oxidized

form (+5) (Table 11.2). As will be elucidated below, the primary implications of these observations are that (i) nitrification is an obligatorily aerobic process and (ii) oxidation of ammonium results in acidification of the soil environment.

11.6.1 Identity of Bacterial Species that Nitrify

All nitrifiers are closely related by their specialized biochemical reactions. All oxidize reduced nitrogenous compounds for energy and fix carbon dioxide for their carbon source. The primary taxonomic difference at the genus level is based on cellular morphology. Common ammonium oxidizers, nitrite oxidizers, their source and morphology are listed in Table 11.3. Note that the ammonium oxidizers are distinguished by the prefix *Nitroso-* for the genus name whereas the nitrite oxidizers have the comparable prefix *Nitro-*.

Some confusion may develop in study of classical literature due to the impact of past dogma on our concept of the role of these various species in soil. Prior to the early 1980s, it was commonly held that of the five nitrifiers, only *Nitrosomonas* spp. and *Nitrobacter* spp. occurred commonly in soil. This assumption led to the conclusion that these two genera were *undoubtedly* the primary nitrifying chemoautotrophs in soil ecosystems. Therefore, when population densities of ammonium oxidizers were estimated, the numbers were presented as populations densities of *Nitrosomonas*. Similarly, the epithet *Nitrobacter* was used globally for nitrite oxidizers. With the advent of the use of fluorescent antibodies to quantify nitrifier populations in soil at the species level, it has become evident that a variety of different species are found in soil systems (e.g. Belser and Schmidt 1978a, b; Fliermans et al. 1974; Stanley and Schmidt 1981). As more specific enumeration procedures such as polymerase chain reaction (PCR) (e.g. Navarro et al. 1992) and fluorescent antibodies specific for various genera of nitrifiers are applied to the study of the diversity of nitrifier populations in soil, an improved understanding

Table 11.2 Oxidation state of nitrogen compounds associated with soil nitrogen cycling

Compound	N oxidation state
NH_4^+	−3
NH_2OH	−1
NO	+2
N_2O	+1
NO_2^-	+3
NO_3^-	+5

Table 11.3 Common species of autotrophic nitrifiers, their sources, and morphologies

Bacterial species	Source	Morphology
Ammonium oxidizers:		
Nitrosomonas europea	Soil, water, sewage	Ellipsoidal, short rods
Nitrosospira briensis	Soil	Spiral
Nitrosococcus oceanus	Marine	Spherical
Nitrosococcus mobilis	Marine	Spherical
Nitrosococcus nitrosus	Marine, soil	Spherical
Nitrosolobus multiformis	Soil	Lobate, pleomorphic
Nitrosovibrio tenuis	Soil	Comma shaped
Nitrite oxidizers:		
Nitrococcus mobilis	Marine	Spherical
Nitrobacter gracilis	Marine	Short rods
Nitrobacter winogradski	Soil	Short rods
Nitrobacter agilis	Soil, water	Short rods

of the variety of these organisms *in situ* will emerge. At this point, it is evident that sufficient variation in the identity of active nitrifiers in soils occurs to justify the use of the epithets ammonium and nitrite oxidizers in place of the more specific genera designations.

11.6.2 Benefits to the Microorganism from Nitrification

The primary organisms responsible for ammonium oxidation to nitrate in soil are chemolithotrophs (see Section 11.6.6 for discussion of the possible role of heterotrophic nitrifiers in soil). The organisms derive their energy from the oxidation of the nitrogen atom. Conversion of ammonium to nitrite yields 66 kcal per mole whereas nitrite oxidation to nitrate produces 20 kcal. The recovery of this energy by the microbes ranges from about 5% to 15%. Thus, large quantities of ammonium or nitrite must be oxidized for each carbon fixed by the microbes. Ammonium oxidizers typically oxidize between 14 and 70 ammonium-nitrogen per carbon incorporated into cellular biomass whereas between 76 and 135 nitrite-nitrogen must be oxidized for the comparable task.

11.6.3 Quantification of Nitrifiers in Soil Samples

Difficulties associated with culture of the bacterial species involved in nitrification have resulted in the development of indirect culture methods and procedures

for direct observation of cells within the soil sample or in extracts. Historically, the two most commonly employed techniques for quantifying soil nitrifiers have been the most probable number method using nitrifier-specific growth media and direct enumeration of specific organisms using fluorescent antibodies. As marker genes for these organisms are identified, PCR-based procedures will provide a clearer estimation of these populations. Examples of research applying 16S rDNA gene analysis to study nitrifier population diversity include Hastings et al. (1997), Kowalchuk et al. (1997), and Stephen et al. (1996).

Two requirements of the most probable number method are that (i) media and growth conditions must be used which allow any and all nitrifying cells present in the soil sample to grow to a population density which can be detected and (ii) the cells must be sufficiently separated from any particulate matter in soil that each cell will be individually and independently dispersed in the diluent (that is, nitrifier cells and not soil particles must be enumerated). No most probable number technique for quantification of nitrifiers meets either of these requirements. All media are selective. Only a portion of the nitrifier population is able to grow. This situation is the most likely reason for the development of the central dogma cited above in that the dominant species growing in the commonly used most probable number media are *Nitrosomonas* sp.

A further complication associated with essentially all procedures based on selective media is that the more rapidly growing organisms limit or preclude development of populations of more fastidious organisms. Support for the role of this phenomenon with nitrifiers is seen in the work of Matulewich et al. (1975) where it was found that the much shorter incubation times commonly employed for nitrifier assays (e.g. Alexander and Clark 1965) did not allow for maximal population development. Incubation times of up to 55 and 100 days were required for ammonium and nitrite oxidizers, respectively. Difficulties associated with using the longer incubation periods include delays in data acquisition as well as the generally encountered problems associated with the evaporation of the liquid growth media. With longer incubation times, the increased salt concentration in the growth media due to the loss of water may itself induce artifacts in population selection. Therefore, analysis of data procured through this procedure must always be predicated on an appreciation of the limitations imposed by the selectivity of the media, artifacts resulting from the long incubation times necessary for population development, and the failure of some investigators to use sufficient incubation periods for their studies. These inherent methodological problems are enhanced by the low statistical precision inherent in such procedures. The latter limitation precludes utilization of the procedure for studies where detection of slight changes in population densities is necessary.

In light of these methodological problems, the question may be asked, "Why is the most probable number procedure still the most commonly used to estimate field populations of nitrifiers?" Perhaps, even more crucial is the question, "Why

is its continued common usage likely?" The answer to both of these questions relates to the practicality of the procedure. Most probable number assays of nitrifiers are still the only methods available to most investigators in the field.

All of the problems inherent in the most probable number procedures are overcome with the use of fluorescent antibody techniques. Data are produced rapidly, specific populations are quantified, and small changes in populations of the nitrifiers are easily measured. Yet, as with any scientific procedure, some methodological limitations of the fluorescent antibody technique govern the wide implementation of the procedure. A major barrier to its application is the availability of equipment necessary for the assays. Whereas most probable number incubations can be conducted with equipment available in essentially any field microbiology laboratory, fluorescent antibody procedures require at least the availability of a fluorescent microscope. Should in-house production of the species-specific antibodies be necessary, the investigator will also need to have the necessary animal care facilities. Specific data interpretation difficulties associated with the use of fluorescent antibodies are that (i) many serotypes of autotrophic nitrifiers occur (thus, large numbers of fluorescent antibody types may have to be used to quantify the total nitrifier population), and (ii) it is difficult to isolate and purify nitrifiers, which is a necessary prerequisite for antibody production. This latter limitation assures that noncultured nitrifiers will not be detected by this method.

11.6.4 Discrepancies between Population Enumeration Data and Field Nitrification Rates

As indicated above, the energy yield from oxidation of ammonium and nitrite and the free energy efficiency for the process are known. Thus, it is reasonable to assume that some relationship between the amount of nitrate produced in a soil system and the population of nitrifiers detected therein should occur. Unfortunately, the most common observation is that the population density of autotrophic nitrifiers in soil samples is insufficient to yield the nitrate concentrations detected. A number of reasons have been proposed to explain the difficulties.

- *Inherent inaccuracies of the most probable number procedure*: These include the fact that in many situations, total nitrifier populations are underestimated because of their inability to grow in the selective media utilized as well as the imprecision of the procedure.
- *Noncoincidence of nitrifiers and nitrate production*: The nitrate present in the soil sample could have been produced in the past and the population of nitrifiers responsible for its production subsequently declined.
- *Errors in estimating growth yields*: The growth yields in culture used to estimate population production per unit of ammonium or nitrite oxidized probably differ from those yielded in soil.

- *Imported nitrate*: The nitrate detected in a soil sample may not have been produced *in situ*. Nitrate moves freely in the soil profile. The nitrate detected in a particular sample of soil could have been produced elsewhere and transported into the soil sample with water infiltration.

Each of these explanations for the fact that the nitrifier population detected in soil samples is frequently less than 10% of that necessary to produce the nitrate present in the samples is plausible.

11.6.5 Sources of Ammonium and Nitrite for Nitrifiers

Ammonification is the primary *in situ* source of ammonium in nonfertilized soils. External ammonium inputs include point sources such as sewage or comparable waste substances amended to soil as well as nonpoint sources such as nitrogenous fertilizers leaching from or applied to cropped lands. Continuous enrichment of nitrifiers may occur downstream from a sewage plant or intermittent population stimulation may occur from irregular flow from cropped soils due to variation in rainfall patterns.

Any nitrite found in soil is presumed to arise from ammonium oxidation. Under normal situations, nitrite does not accumulate in soils. Thus, the limiting factor in nitrite oxidation to nitrate in most soils is the conversion of ammonium to nitrite (low levels of ammonium are detected in most soils). The nitrite is usually converted to nitrate as rapidly as it is formed. Under alkaline conditions, nitrite may accumulate. This results from the fact that ammonia is toxic to *Nitrobacter* sp. An equilibrium between ammonium and ammonia normally exists in soils, with ammonium predominating in neutral and acidic conditions and ammonia predominating under alkaline conditions. Thus, under alkaline conditions, *Nitrobacter* spp. are inhibited but the ammonium oxidizers remain active. Hence, nitrite accumulates. This phenomenon also occurs in microsites when anhydrous ammonium is added to soil.

Nitrite oxidizers may also derive a portion of their growth substrate from the reduction of nitrate (e.g. Belser 1977). In a flooded system, nitrate may be reduced to nitrite in anaerobic microenvironments. The nitrite may then diffuse to aerobic sites where the nitrite oxidizers can convert it to nitrate. In these situations, populations of nitrite oxidizers would be elevated in comparison to the ammonium oxidizers.

11.6.6 Environmental Properties Limiting Nitrification

In contrast to the situation with both carbon and nitrogen mineralizers, where a wide diversity of soil microbes are capable of catalyzing the process, the kinetics and occurrence of nitrification in soil reflect the fact that a limited number of bacterial species are involved in the process. Primary concerns in predicting the extent and rate of nitrification in an ecosystem are substrate availability, temperature, pH, oxygen tension, and soil moisture.

Substrate availability: As indicated above, most soils contain low concentrations of nitrate and ammonium, with detectable nitrite levels occurring rarely (e.g. Jones and Schwab 1993). Thus, it can be concluded that the nitrite oxidizers are generally controlled by the presence of their growth substrate, which indirectly is determined by ammonium availability. Thus, the growth-limiting step for nitrification in general is nitrogen mineralization.

The kinetics of nitrification processes necessarily depend upon population density of nitrifying bacteria and efficiency of nitrifying enzymes. Nitrifiers are highly efficient at recovering ammonium and nitrite from their environment. That is, the enzymes responsible have low Michaelis constants. Properties of the nitrifier community that can be deduced from the reaction kinetics are as follows.

- Nitrate production may increase proportionally to ammonium concentration in the soil sample. In this situation, the ammonium oxidizing enzymes in the existing nitrifier population are substrate limited. Thus, nitrate production in these systems is described by Michaelis–Menten kinetics (e.g. Malhi and McGill 1982).
- Nitrate production in some cases is described by zero-order kinetics. In this situation the indigenous ammonium production is adequate to provide all the substrate necessary to support growth of the indigenous nitrifier population. Energy sources (NH_4^+) and energy consumers (ammonium oxidizers) are in balance. A relatively constant nitrification rate can be anticipated to occur unless this balance is shifted; that is, through an increase in NH_4^+ supply or inhibition of the nitrifier population. Augmentation of the energy supply can induce population growth. Then, the nitrifier population will increase until another environmental factor (e.g. space, oxygen, pH, species interactions) becomes limiting. Amendment of an ecosystem wherein the nitrifier population is controlled by one of these latter site properties rather than growth substrate availability will not induce population growth and thereby deviation from zero-order kinetics will not occur.
- Nitrification may not occur in an ecosystem until several days following amendment. In this case, for some reason a functional nitrifier population has not developed. With amendment of the soil site with ammonium, a nitrifier population slowly develops. An example of this phenomenon was provided in a study of a pine forest soil by Vitousek and Matson (1985).

In conclusion, the *in situ* nitrification rate varies with the degree of saturation of the existent enzymes in the soil system and the environmental limitations controlling nitrifier population development. Changes in the nitrification rate are dependent upon the capacity of the nitrifier population density to increase.

Toxic concentrations of ammonium and nitrite can be reached in axenic cultures of nitrifiers. Generally, normally encountered environmental levels of the nitrification substrates are far below these inhibitory concentrations, but elevated

ammonium concentrations have been shown to inhibit the process. For example, Malhi and McGill (1982), in a study of three Canadian soils, found that concentrations of $200\,\mu g\,g^{-1}$ NH_4^+-N supported rapid nitrification but elevation of the ammonium-nitrogen concentration to $300\,\mu g\,g^{-1}$ inhibited the process. Malhi and McGill suggested that the inhibition could result from a combined effect of low pH and salt content.

Temperature: In the laboratory, ammonium oxidation to nitrite occurs at temperatures from near freezing to 65 °C. Nitrite oxidation occurs from near freezing and is completely inhibited at about 40 °C. The latter process has an optimum generally reported to be between 30 and 35 °C. Since nitrification occurs in tropical and subtropical soils at temperatures far above the limits for nitrite oxidation, some compensation must occur in the microbe's native environment that is not provided under axenic culture.

pH: In culture, both the rate of nitrification and the number of nitrifiers decline below a pH of about 6.0. Negligible nitrification is detected below a pH of 5.0–5.5 The generally detected optimum for this process is between 6.6 and 8.0. This observation has led to the dogma that autotrophic nitrification cannot occur in acidic environments yet, it can readily be shown that nitrification does occur in acid soils, such as commonly encountered in forest soils. Explanations of this discrepancy between the laboratory and "real-world" observations include (i) microsite variation in soil pH, (ii) invoking the possibility of the occurrence of nonculturable, acidophilic, autotrophic nitrifiers in these soils, (iii) selection of heterotrophic nitrifier populations under these acidic conditions, and (iv) adaptation of the growth properties of commonly encountered nitrifiers to accommodate the acidic conditions.

Each of these hypothesized mechanisms for nitrification in acid forest soils may be operative under some situations but none is totally satisfying. (See Dommergues et al. 1978 for a review of the pros and cons of these mechanisms.) For example, the possibility of nonculturable, acid-loving organisms is plausible, and some acid-loving autotrophic nitrifiers have been cultured (e.g. Hankinson and Schmidt 1988). The presence of humic acid has also been shown to provide some relief of pH inhibition of nitrification in soil columns (Bazin et al. 1991). The postulation of more neutral microsites in otherwise acidic soils is plausible in soils whose predominant pH is near the limit for autotrophic nitrifiers, but prediction of the occurrence of such islands of neutrality in extremely acidic soils is implausible. The potential for the formation of microaggregates of nitrifying bacteria in acidic soils is interesting and a potentially useful resolution of this laboratory-field anomaly (De Boer et al. 1991, 1993). The size of the aggregates is limited by the penetration depth of the oxygen necessary for this obligatorily aerobic process.

The most commonly encountered (and perhaps debated) interpretation of the field data relating to nitrification in acidic soils involves the role of heterotrophic nitrifiers. Heterotrophic nitrification is the oxidation of reduced nitrogen forms to

nitrite or nitrate by heterotrophic bacteria and fungi. The reduced nitrogen can be either organic or inorganic. Heterotrophic nitrification can be easily demonstrated in culture. Large numbers of soil bacteria, actinomycetes, and fungi have long been known to exist in soil which are capable of oxidizing ammonium to nitrite or nitrate (e.g. Doxtander and Alexander 1966; Eylar and Schmidt 1959; Schimel et al. 1984; Tate 1977; Verstraete and Alexander 1973), but their function in native soil ecosystems at rates to affect significantly *in situ* nitrate concentrations is questioned. Ammonium or organic nitrogen oxidation by these organisms generally occurs after the active growth phase of the organism in media with a low carbon/nitrogen ratio. More nitrogen is usually present than the organisms need for growth. The quantities of nitrate or nitrite produced are small compared to those produced by the autotrophic population (less than 1 vs 2000 or greater $\mu g\,mL^{-1}$ solution for the heterotrophs and autotrophs, respectively). Since the oxidation of the reduced nitrogen occurs subsequent to replication of the heterotrophic cells, it is unlikely that the organisms are recovering the energy from the oxidation. Although there are reports of the existence of large populations of heterotrophic microbes in some ecosystems, their environmental significance is unknown.

Oxygen tension: Nitrification is an obligatorily aerobic process. Molecular oxygen is directly incorporated into the final product. Therefore both soil moisture and soil structure impact the nitrification rate indirectly through control of oxygen diffusion. Nitrification can occur at oxygen concentrations as low as $0.3\,\mu g\,mL^{-1}$, thus the key to the occurrence of this process in waterlogged or otherwise diffusion-limited systems is the availability of a means for oxygen import. Influx of oxygen into soils where it would normally not be found can be through such processes as mass movement of oxygenated water into flooded soils or even transport of air through the plant into the rhizosphere, as occurs with rice plants.

Moisture: As indicated above, limitations of nitrification in elevated moisture situations are derived primarily from the indirect control of availability of molecular oxygen by its diffusion rate in water. At the other extreme, desiccated soils, preclusion of nitrification in extremely dry soils, and the direct proportionality of nitrification rate with moisture level between this totally inhibitory level and moisture saturation results from the fact that the soil microbial community is essentially aquatic. That is, microbes require a thin film of water on the soil particles within which to respire and grow. In the absence of this layer of water, no nitrification can occur. As the thickness of the coating of water on soil particles increases, the "aquatic" soil nitrifier bacterial population becomes active.

Other limitations to nitrification: Two site characteristics with potential to alter soil nitrification potential which should be considered in land reclamation or management plans are metal contamination and salinity. Both have been demonstrated to have a significant impact on nitrification rates.

Liang and Tabatabai (1978) assessed the inhibition of nitrification by a variety of elements including silver, mercury, cadmium, nickel, arsenic, chromium,

boron, aluminum, selenium, molybdenum, manganese, lead, cobalt, copper, tin, iron (II and III), and zinc. Soil pHs ranged from 5.8 to 7.8, clay contents from 39% to 50%, and organic matter from 2.58% to 5.45%. When added at a concentration of $5\,\mu mol\,g^{-1}$ soi,l all 19 metals inhibited nitrification, but the amount of inhibition varied with metal added and the soil. The potential exists for adaptation of the microbial population to the toxicant in situations where the active microbial population is not killed. This possibility was suggested by a report by Morrissey et al. (1974) when they recorded an initial inhibition of nitrification in a Cheshire fine sandy loam soil by cadmium, chromium, copper, lead, manganese, nickel, and zinc and a subsequent adaptation to the presence of the metal over a several-week period. Thus, these workers concluded that although with elevated metal concentrations a transient inhibition was observed, in the long run there was little significant inhibition of nitrification by the metals in their system.

McClung and Frankenberger (1985) noted that increased salinity generally decreased nitrification rate in three diverse soils. Inhibition of the nitrification rate varied from 8% to 83%, dependent upon the soil type and the nature of the salt added (Na_2SO_4, NaCl, or $CaCl_2$). Generally, the sulfate salt was less inhibitory than were the chloride salts. As with the metal-contaminated soils cited above, the potential exists for microbial adaptation to increased salt content. Somville (1984) found that freshwater nitrifiers were capable of adapting to increased salinity in an estuarine environment.

11.7 Concluding Observations: Control of the Internal Soil Nitrogen Cycle

In nonmanaged or minimally managed soil ecosystems (e.g. native forests, "wild lands"), nitrogen cycling could be described as being in balance. That is, organic nitrogen is mineralized to ammonium, a portion is nitrified to nitrate, and most of the mineral nitrogen is returned to biomass. This balance does not imply that no mineral nitrogen is lost from the site. Aside from denitrification processes (as discussed in Chapter 14), some nitrate can be leached below the root zone through soil-saturating rain falls. In balanced systems, this small nitrogen loss is balanced by atmospheric inputs and nitrogen fixation (see Chapter 12).

Greater potential for disruption of the balance between nitrogen mineralization and incorporation into nascent biomass occurs in managed sites as exemplified by systems amended with external supplies of organic matter or receiving significant mineral fertilizer inputs. In these situations, the internal nitrogen cycle could be said to have been disrupted or skewed by the anthropogenic intervention. Rates of soil biological processes generally adjust in these situations (usually through enhanced biomass production, perhaps through increased denitrification), but

the potential also exists for enhanced negative ecosystem impact. Since ammonium is relatively nonmobile in the soil profile (Stevenson 1986), most environmental contamination difficulties are derived from the synthesis of nitrate, i.e. nitrification.

The process of nitrification in soil can be likened to a double-edged sword. To maximize crop production and plant community development in reclaimed sites, fertilization with nitrogenous fertilizers is desirable, if not essential. Nitrification of this added nitrogen is necessary to maximize plant incorporation, but this oxidation of ammonium to nitrate greatly increases the mobility of this essential nutrient. Hence, nitrate may be leached into groundwaters or transported through overland flow into surface waters. A desirable soil substituent is transformed into a water contaminant.

The best means to reduce the environmental impact of nitrification in a managed system is an attempt to maintain nitrate availability at the level required by the growing plant. This control is generally achieved through the use of slow-release fertilizers or nitrification inhibitors.

Ammonium concentration is the primary nutrient limitation of nitrification in soil. Once the ammonium is formed, it is rapidly transformed to nitrate – a trait desirable in balanced systems where plant biomass synthesis is tightly linked to organic matter mineralization. Such is not the case in fertilized sites. To slow ammonium release from fertilizers, the fertilizer particles (for example, urea) can be coated with a substances that slowly dissolves in soil, thus gradually releasing the ammonium or ammonium precursor. This objective is commonly achieved through the use of slow-release fertilizers or timed mixing of plant growth nutrients with irrigation water (e.g. fertigation of turf or golf grasses or drip irrigation systems for row crops). Dissolution of fertilizer nitrogen can be achieved by variation of fertilizer particle size, coating of the particles with slowly dissolving or metabolized substances such as sulfur-coated urea (e.g. Landschoot and Waddington 1987; Severson and Mahler 1988) or by incorporation of the fertilizer in polymers (e.g. Gandeza et al. 1991), which are commonly used in urban systems for yard and turf fertilization or in agricultural systems, as well as through the use of urease inhibitors (e.g. Bremner and Douglas 1971; Gould et al. 1978; Krogmeier et al. 1989). Urease is an enzyme that produces ammonium through the hydrolysis of urea. Similarly, a variety of compounds have been shown to be efficient and effective inhibitors of the conversion of ammonium, once it is produced, to nitrate (e.g. Baldwin et al. 1983; Belser and Mays 1980; Belser and Schmidt 1981; Bremner and McCarty 1993; Goring 1962; McCarty and Bremner 1986).

It must be noted that the role of plant-derived toxicants in the inhibition of nitrification in soil is questionable, in that although these compounds may inhibit nitrifier activity in culture, there is little evidence supporting their function at concentrations naturally detected in soil systems (Bremner and McCarty 1993).

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12

Nitrogen Fixation

The Gateway to Soil Nitrogen Cycling

A reliable source of fixed nitrogen is an obligatory requirement for sustaining all biomass components, above and below ground, in terrestrial ecosystems. In sites with significant accumulations of organic matter, the majority of this nutrient is provided by the internal cycling of the nitrogen between organic and plant available inorganic pools as was described for climax forest ecosystems in Chapter 11. In contrast, the biological communities of more nutrient-limited soil ecosystems may be totally reliant upon external sources for support of living community development. Such situations are characteristic of pioneer soil sites, as exemplified by volcanic soils, which contain essentially no or minimal fixed nitrogen pools. Although there is a clear difference between climax and pioneer systems, care must be taken in not undervaluing the importance of fixed nitrogen inputs in either system. Due to the inevitable losses of fixed nitrogen from all soils, some external supply of fixed nitrogen is always required to maintain long-term ecosystem productivity. Otherwise, soil fixed nitrogen reserves would slowly decline to the level that biomass productivity is reduced or even precluded. Therefore, it can be concluded that a portion of the foundation for sustained productivity of soil-based systems is insured by the replenishment of the fixed nitrogen pool, in part, through the function of a nitrogen-fixing microbial population *in situ*.

Fortunately for the development of highly productive, sustainable terrestrial systems, fixed nitrogen inputs do generally balance losses. One example of estimates of these losses and inputs was provided by Knowles (1981). Specifically, estimates of these losses of fixed nitrogen from terrestrial ecosystems were indicated to range from 160 to 225 $TG\,yr^{-1}$ but were balanced by inputs of newly fixed nitrogen of 214–262 $Tg\,yr^{-1}$ (Knowles 1981). The rising trend in these numbers due to increased agricultural demands was suggested by Galloway et al. (2008). A summary of the various pathways of fixed nitrogen into and out of land ecosystems in general is demonstrated in Figure 12.1. Primary routes for nitrogen loss from soils are volatilization, runoff, and denitrification. By far the largest nitrogen

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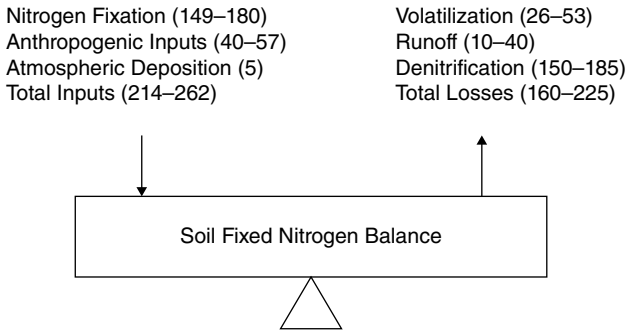

Figure 12.1 Major nitrogen inputs and losses contributing to the soil fixed nitrogen balance. Values in parentheses are Tg N yr^{-1} as estimated by Knowles (1981).

source in noncultivated soils is biological nitrogen fixation. In general, fertilizer application (anthropogenic input) is a distant second. This conclusion is based in part on the observation that the vast majority of the world's agricultural soils do not receive appropriate inputs of fertilizer in relationship to the stresses resulting from the food demands of ever growing populations. Understandably, most of the anthropogenically fixed nitrogen entering the soil ecosystem (estimated to be 30 Tg yr^{-1} by Burns and Hardy 1975) is applied to agricultural and urban soils (i.e. lawns and recreational areas).

Interestingly, a less obvious observation is that due to the intensive management of nitrogen-fixing crops, the majority of the biologically fixed nitrogen enters the land-based nitrogen reserves in cropped lands (Burns and Hardy 1975; Galloway et al. 2008). True, intensive agricultural systems have, for the most part, been sustained through liberal use of industrially fixed nitrogen, but societal as well as environmental concerns are dictating a return to utilization of biological nitrogen fixation processes to replace at least a portion of the quantities of industrially fixed nitrogen used in crop production. Most commonly, this entails cultivation of legumes, crops commonly involved in symbiotic associations with nitrogen-fixing bacteria. Similar plant–microbe partnerships involved in nitrogen fixation are actinorhizal associations. In contrast to the legume interactions, which tend to be identified with temperate agricultural systems, actinorhizal symbioses are more generally linked to native or "wild land" situations. (It must be noted that many of the vast number of underappreciated legumes are tree species, which therefore would be associated with less managed ecosystems.)

With legume crops, the greatest gain in fixed nitrogen results from return of all new plant biomass to the soil. Removal (i.e. harvesting) of aboveground portions of the plant leads to little increase in soil nitrogen. Some benefit results from retention of the legume root system within soil (Alexander 1977) but the bulk of

the fixed nitrogen is contained within the harvested crop. Therefore, the best situation for cultivated legumes that can be anticipated would be retention of sufficient fixed nitrogen through incorporation of the root systems alone to maintain or minimize fixed nitrogen losses. In contrast to this situation with cropped legumes (e.g. soybeans, beans, alfalfa, and peas), a less appreciated, perhaps even more beneficial situation results from growth of leguminous trees. General soil fertility gains are realized for growth of the nitrogen-fixing trees (e.g. Budowski and Russo 1997; Jonsson et al. 1996; Sanginga et al. 1996).

These introductory comments reveal the critical nature of nitrogen fixation in maintenance of native as well as managed ecosystems. Consideration of the balance between fixed nitrogen supplies, losses, and inputs in a soil ecosystem is especially important for the development of management or reclamation plans for damaged or mismanaged soil sites. Thus, this chapter is presented with the primary objective of evaluating nitrogen fixation as a process and of elucidating the nuances of variation of ecosystem properties on nitrogen fixation rates in managed and native soil systems.

12.1 Biochemistry of Nitrogen Fixation

An indepth evaluation of the biological, biochemical, and genetic aspects of biological nitrogen fixation exceeds the purview of primary interest of most soil microbiologists. Yet, a foundational understanding of some essential properties of the enzymes and organisms involved is necessary to comprehend the soil ecosystem that controls the dynamics of nitrogen fixation in native ecosystems. Primary traits of biological nitrogen fixation controlling the yields of fixed nitrogen in native soils and the kinetics of this production to be considered herein are the complexity of the enzyme (nitrogenase) catalyzing the reduction of dinitrogen (N_2) to ammonium, the quantities and sources of the energy required to drive the process, and the diversity of microbes responsible for the process. For more detailed analyses of the biochemical and genetic aspects of nitrogen fixation processes, see basic biochemistry and general microbiology textbooks and reviews of the topic (e.g. Brewin 1991; Burris 1991; Caetano-Anollés and Gresshoff 1991).

12.1.1 The Process

Dinitrogen, because of the triple bond between the two nitrogen atoms, is a nearly inert molecule with respect to the metabolic potentialities of the biological community. The only enzymologically catalyzed reaction involving dinitrogen is accomplished by a limited number of bacterial strains. As will become evident when individual microbial genera are discussed, these nitrogen-fixing bacteria

constitute a highly diverse physiological group with the capacity to reduce dinitrogen to ammonium as their primary common trait. It should also be stated that the ability to transform dinitrogen to ammonium is limited to bacteria; that is, thus far no fungi, plants, or animals have been found to possess this capability.

In nitrogen fixation, the nitrogen atom is reduced from its most oxidized state (N_2) to its most reduced form (NH_4^+) by the following reaction:

$$N_2 + 8H^+ + \text{energy} \rightarrow 2NH_3 + H_2$$

Although the nitrogen atom passes through a number of oxidation states in between dinitrogen and ammonium, no free intermediates between dinitrogen and ammonia are produced. All intermediates are retained within the nitrogen-fixing bacterial cell and associated with the enzyme catalyzing the reaction (i.e. nitrogenase). Thus, the only direct product of nitrogen fixation encountered by soil microbes is ammonium.

Energy consumption during nitrogen fixation: Considering the bonding energy associated with the triple bond of dinitrogen, it is reasonable to conclude that nitrogen fixation is an energy-intensive process. Under ideal conditions, a minimum of 16 molecules of MgATP are required for each atom of dinitrogen cleaved and reduced. Since ideal conditions are rarely achieved in soil, the quantity of energy required is generally found to be between 20 and 30 molecules of MgATP (Burris 1991). To put this quantity of energy into perspective, recall that the total oxidation of one molecule of glucose yields a maximum of 38 ATP molecules (see Chapter 4). Thus, a microbe fixing nitrogen must devote nearly all of the energy provided by the oxidation of a glucose molecule to the reduction of one dinitrogen molecule to two ammonium ions. The ATP molecules supporting nitrogen fixation are derived primarily either directly from photosynthetic processes (nitrogen-fixing photoautotrophs) or from decomposition of organic compounds (heterotrophic metabolism).

Knowledge of this energy requirement allows the soil microbiologist to deduce the sites of activity and the relative importance of the various metabolic groups of nitrogen-fixing bacteria. For example, as will be discussed below, this energy requirement limits the quantities of dinitrogen that can be reduced by microbes catalyzing soil organic matter as their energy source. For maximal conversion of dinitrogen to ammonium, a stable, abundant energy source is necessary. Such reliable supplies occur naturally in the rhizosphere (i.e. photosynthetically fixed carbon contained in root exudates or provided by symbiotic associations) or through direct conversion of light energy for the process (cyanobacteria and photosynthetic bacteria). Therefore the quantity of easily metabolized organic matter in soils in general is rarely sufficient to support high levels of nitrogen fixation.

This energy requirement essentially dictates that the primary sites for nitrogen fixation in soil are located in the rhizosphere. Potential exceptions to this observation

could be soil sites receiving large quantities of fixed carbon; that is, spill sites or systems receiving large quantities of industrial effluents. For example, Neilson and Sparell (1976) isolated nitrogen-fixing enterobacteria from paper mill process waters. In conclusion, in native ecosystems, highest nitrogen fixation contributions to soil ecosystems are associated with situations where energy inputs are provided by photosynthesis (nitrogen fixation by blue-green bacteria) or through transfer of plant photosynthate directly to nitrogen-fixing bacteria or actinomycetes (symbiotic nitrogen fixation).

Hydrogen production and hydrogenase activity: A related concern involving the stoichiometry of reduction of dinitrogen to ammonium involves the inefficiency associated with loss of reducing power (H^+) through the formation of hydrogen gas. It appears to be logical that the most efficient process would be the incorporation of all reducing power into the nitrogenous product but that does not occur. Formation of hydrogen gas appears to be illogical also from the fact that hydrogen is a specific competitive inhibitor of nitrogenase. Although there has been considerable research involving optimization of utilization of reducing power in dinitrogen reduction, the synthesis of hydrogen gas appears to be an obligatory part of the nitrogen fixation reaction (Burris 1991).

The quantities of electron flow lost to the microbe through hydrogen ion reduction can be significant. For example, nodules formed by *Rhizobium meliloti* or *R. trifolii* on their respective hosts have been shown to lose at least 17% of their electrons through this mechanism (Ruiz-Argüeso et al. 1979). The mean conversion of reducing power to hydrogen gas for each of these organisms was considerable greater than this minimum value. For the alfalfa nodulator *R. meliloti*, the average proportion of electrons lost was 25%. The comparable value for the clover-modulating organism (*R. trifolii*) was 35%.

Although there appears to be no way of preventing the formation of this hydrogen gas during nitrogen fixation, a means of improving the efficiency of the nitrogen-reducing process for the microorganism is to recover the energy contained in hydrogen gas. Hydrogenase is an enzyme that catalyzes the oxidation of hydrogen gas with water as the final product. This oxidative reaction is coupled with ATP formation or with reduction of ferredoxin or flavodoxin. These electron acceptors are of particular interest since their reduction can lead to increased ammonium ion yields. The electrons transferred to ferredoxin and flavodoxin are returned to the dinitrogen reductase portion of the nitrogenase enzyme. Examples of the occurrence of hydrogenase activity in nitrogen-fixing bacteria include *Rhodopseudomonas capsulata* (Colbeau et al. 1980). High hydrogenase activities were also found *Bradyrhizobium japonicum* (Keyser et al. 1984; Merberg and Maier 1983), and *Rhizobium leguminosarum* (Nelson and Salminen 1982). The proportion of each of the strains of the species producing hydrogenase is extremely variable. For example, in the study conducted by Keyser et al. (1984),

a small portion of the *B. japonicum* strains produced hydrogenase (i.e. were Hup^+). Of 972 *B. japonicum* isolates from 65 soybean fields located in 12 states (USA) studied, only 20% produced hydrogenase. Frequency of occurrence of these hydrogenase producers varied considerably between strains. None of the isolates in serogroup 135 were Hup^+ whereas 93% were positive in serogroup 122.

12.1.2 The Enzyme, Nitrogenase

Reduction of dinitrogen to ammonium in nitrogen-fixing bacteria is catalyzed by an enzyme system consisting of two distinct proteins: dinitrogenase (also known as MoFe protein or protein I) and dinitrogenase reductase (alias Fe protein or protein II). Dinitrogenase is a large protein (220–240 KDa) that binds to and reduces dinitrogen. Dinitrogenase reductase transfers the electrons to dinitrogenase.

Dinitrogenase and dinitrogenase reductase are highly conserved proteins between the various strains of nitrogen fixers. This fact is supported by the observation that purified dinitrogenase and dinitrogenase reductase produced from a variety of non-related bacterial species can be combined to produce active nitrogen fixation. Thus, a limited set of basic properties can be attributed to all nitrogenase enzymes.

All nitrogenases are extremely oxygen sensitive. Since nitrogen fixation is catalyzed by strictly anaerobic bacteria, facultative aerobes, microaerophilic organisms, and strictly aerobic bacteria, a major proportion of the organisms involved must possess a mechanism to protect the oxygen-labile protein. Two adaptive mechanisms to protect nitrogenase are available to the microbes. Either a means can be developed that results in the exclusion of molecular oxygen from sites of active nitrogen fixation or nitrogenase molecules inherently resistant to oxygen can be selected.

At least seven individual means for exclusion of molecular oxygen from the environment of nitrogenase enzymes have been observed.

- *Respiratory protection*: The rate of consumption of oxygen by individual cells may be enhanced so that the oxygen in the microenvironment is reduced to acceptable levels. For example, *Azotobacter* sp. cells have been observed to oxidize more fixed carbon in the presence of oxygen than is needed to satisfy microbial energy requirements. This protective mechanism appears to be operative in a variety of free-living organisms, including such metabolically diverse organisms as *Azotobacter vinelandii* (e.g. Shah et al. 1973) and cyanobacteria (e.g. Murry et al. 1984; Peschek et al. 1991).
- *Conformational protection*: An alternative to developing an enzyme that is inherently resistant to oxygen is to develop a nitrogenase that changes to a protective conformation in the presence of oxygen. This has been observed with *Azotobacter*. In the protected state, nitrogen fixation is totally precluded, but the

activity rapidly returns when the oxygen tension is reduced to acceptable levels. This alteration of nitrogenase structure results from association of a small protein (approx. 24 000 Da molecular weight) with the nitrogenase protein.

- *Oxygen regulation of nitrogenase synthesis*: Nitrogenase is a complex of large protein molecules. Synthesis of these molecules thus requires expenditure of significant portions of the cell's energy resources. One means of protection of the nitrogen-fixing apparatus is to prevent its production under unfavorable conditions. Molecular oxygen represses nitrogenase synthesis in *Klebsiella pneumoniae*, *Azotobacter chroococcum*, and some strains of rhizobia.
- *Gum production*: For oxygen to interfere with dinitrogen reduction, the inhibitor and susceptible enzyme must interact. Nitrogenase can be protected by production of a physical barrier to molecular oxygen. Gums or polysaccharides accumulated external to the cell wall may provide such protection. Microorganisms may synthesize extracellular polysaccharides that would reduce the diffusion rate of oxygen in the vicinity of the cell wall. This has been proposed to occur for *Azotobacter* spp. and *Derxia gummosa*.
- *Heterocyst production*: Nitrogen fixation can be an especially incompatible process in photosynthetic organisms in that molecular oxygen is a product of carbon fixation in aerobic, nitrogen-fixing cyanobacteria. With these organisms, the oxygen-labile enzymes may be isolated in structures with thick cell walls (heterocysts) to reduce contact of nitrogenase with molecular oxygen. (For further discussion of the protection of nitrogenase in cyanobacteria, see Fay 1992 or Yoon and Golden 1998.)
- *Leghemoglobin production*: In *Rhizobium*–legume nodules, molecular oxygen is complexed by substances with high oxygen affinities in order to reduce intracellular oxygen concentrations. A hemoglobin-like molecule (leghemoglobin) is synthesized in the cytosol around the packets of bacteria. This complexing of molecular oxygen results in reduction of oxygen at the bacteroid surface to about 10 nM. Further protection of nitrogenase in the bacteroids is provided by a terminal oxidase system that has an unusually high affinity for molecular oxygen.
- *Migration to suitable environment*: Aerotactic organisms may migrate to positions in their microenvironment suitable for nitrogen fixation. This has been observed with *Azospirillum* spp. (Barak et al. 1982).
- *Other mechanisms*: In aquatic ecosystems, as exemplified by cyanobacteria associated with rice cultivation, nitrogen-fixing cells may be protected by clumping of the cells or oxygen-generating and nitrogen-fixing processes may be separated temporally. For example, cyanobacterial cells have been shown to form clusters. Molecular oxygen consumption on the surface of the cluster is sufficient to reduce oxygen concentrations inside the grouping to noninhibitory levels.

12.1.3 Measurement of Biological Nitrogen Fixation in Culture and in the Field

Accurate quantification of fixed nitrogen inputs into terrestrial systems due to the biological nitrogen fixation is difficult to achieve. Even though large quantities of dinitrogen are reduced to ammonium by the soil biological community annually (e.g. Knowles 1981), the actual change in quantities of fixed nitrogen in individual soil microsites is relatively small compared to the background concentrations of both ammonium and dinitrogen, which are common chemical components of both soil air and water as well as the general atmosphere. This situation necessitates sensitive and accurate measurement of small changes in reactants and products. Such procedures for use with complex soil samples are of limited availability.

A variety of techniques adaptable for quantification of dinitrogen or ammonium ions in terrestrial ecosystems are available. Unfortunately, their use to evaluate changes in fixed nitrogen concentrations in native soil samples or in growth media of axenically grown microbial cultures is problematic. To a large degree, use of direct measurements of changes in ammonium or dinitrogen concentrations for quantification of fixed nitrogen production yields equivocal data. It is not unusual for gains or losses of these nitrogenous substances in soil samples where nitrogen fixation is actively occurring to be less than or approximately equal to the intrinsic variability of the procedure used to quantify them. Thus, the sought after value is obscured within the background "noise" associated with the data (i.e. the standard deviation or standard error of the mean of the results).

To overcome the methodological limitations imposed by the ubiquity of dinitrogen and ammonium, a variety of indirect as well as more direct procedures have historically been employed to estimate quantities of nitrogen fixed in culture and in soil samples. Methods utilized have included growth in microbiological media lacking a fixed nitrogen source (based on the assumption that any increase in levels of fixed nitrogen results from nitrogen fixation), assessment of changes in the distribution of fixed nitrogen in known soil nitrogen pools (nitrogen balance studies), tracing movement of nitrogen atoms with ^{15}N-labeled substrates, and use of acetylene reduction as an indicator of nitrogen fixation capacity.

Nitrogen-free cultivation and nitrogen balance procedures: Two apparently logical procedures for assessing nitrogen fixing potential are (i) determination of the capacity of a microorganism to grow in media lacking a source of fixed nitrogen and (ii) assessment of the changes in nitrogen pools in a soil site. These were the most commonly used early methods for assessing nitrogen fixation potentials. Unfortunately, both yield equivocal results, at best. These techniques, although applied to very different systems (field soil sites or samples thereof and defined microbial culture media), are examined together because they are both supported by the same faulty assumption, which is that all nitrogen inputs and losses in a soil sample or a culture medium are understood and can be accurately quantified.

Thus, changes in both quantities of inorganic fixed nitrogen in the field or the amount of the microbial biomass produced in laboratory cultures were assumed to have resulted from biological nitrogen fixation. The tenets of this assumption are rarely achieved in either the field or the laboratory.

From the view of assessment of nitrogen-fixing activity of axenically grown microbial cells, it has long been possible to prepare laboratory media with nitrogen-free chemicals and reasonably pure water. Since it is also known that a fixed nitrogen source is obligatory for cell growth and reproduction, it can logically be concluded that if a microbe grows in a growth medium composed of nitrogen-free water and chemicals, it must be fixing nitrogen. Superficially, it appears that there are no other sources of fixed nitrogen. It is not difficult to find a variety of papers published prior to the 1950s documenting nitrogen-fixing capabilities of a variety of bacterial species through use of this method.

Unfortunately, conclusions reached using nitrogen-free growth media for microbial culture are equivocal at best and are more commonly erroneous. Why? Assuming that the fixed nitrogen has been adequately removed from substituents of the growth media or that the quantities of microbial biomass produced exceed those that would be allowed by the trace nitrogenous contaminants of the substituents of growth medium, it would seem apparent that the only other source of fixed nitrogen for microbial replication would be biological fixation. Unfortunately, fixed nitrogen is a common component of the atmosphere of laboratories and incubators. The tendency to disregard this air pollution resulted in a misplaced confidence in the laboratory results. Volatile nitrogenous compounds are easily transferred via the atmospheric route into the nitrogen-free media in laboratory situations. It is easy to envision conditions where a culture of a microbe producing a volatile nitrogenous compound is growing adjacent to a culture containing a nitrogen-free medium. Furthermore, biological products of neighboring cultures are not the only source of contamination for such studies utilizing nitrogen-free growth media. Although scrupulously clean chemical and water were generally employed in preparing for culture of the test microbes, incorporation of ammonium into the microbial culture media easily occurred any time ammonia-based cleaning solvents were used in the laboratory or when a bottle of ammonium hydroxide was opened in the laboratory.

In the more complex field situations, the extent of biological nitrogen fixation has been historically estimated by quantifying changes in quantities of nitrogen contained within the various soil nitrogen pools. Nitrogen balance sheets were prepared. With this method, fixed nitrogen concentrations were measured directly in soil samples. Ammonium was usually extracted from the sample, steam distilled and titrated. Nitrate and nitrite ions were then reduced to ammonium and similarly assayed. Finally, organic nitrogen was converted to ammonium using the Kjeldahl digestion procedure. Two requirements that are basic to any nitrogen

balance-sheet analysis are (i) diligence in analyzing all nitrogen sources and (ii) a capacity to assess accurately small changes in generally sizable nitrogen pools. Neither condition is met in these analyses. Error results from difficulties in quantifying fixed nitrogen losses through denitrification and erosion as well as inputs from atmospheric sources (e.g. inputs of volatized ammonium). The problems associated with quantification of slight changes in preexisting fixed nitrogen pools were discussed above. Applicability of nitrogen balance data to total ecosystems is further reduced by the inaccuracies introduced by extrapolation of data with high standard deviations collected by analysis of a single or a limited number of field samples to account for all nitrogen inputs into the study site. Use of nitrogen balance-sheet type studies is most commonly limited to well-defined systems experiencing major changes in the size of the nitrogen reservoirs, such as the closed system represented by a soil sample incubated in a beaker in the laboratory.

Use of ^{15}N tracers: A nearly ideal means of compensating for the tendency of background nitrogen concentrations to obscure small changes in distribution of atoms among the various reservoirs of soil nitrogen is to trace the movement of ^{15}N-labeled atoms within the system of interest. Sufficient labeled dinitrogen can be added to the soil sample to overcome any difficulties associated with the natural background levels of the nitrogen isotope contained therein. There are no radioactive nitrogen isotopes with a sufficiently long half-life to be practical for quantifying nitrogen fixation in soil environments. The heavy isotope ^{15}N can be used to trace nitrogen transformations in natural soil samples. Assays for ^{15}N in soil nitrogen pools are sensitive and accurate.

The primary assumption underlying use of ^{15}N for quantification of nitrogen fixation is that the labeled atom is utilized by the microbes indiscriminately; that is, the microbes are not capable of differentiating between the ^{15}N label and the more abundant ^{14}N isotope. A further consideration is that an apparent microbial discrimination in nitrogen atom sources must not be imposed on the system by incomplete homogenization of the amended labeled nitrogenous substrate with indigenous soil nitrogen pools. This means that the ^{15}N-labeled dinitrogen molecules must be reduced to ammonium proportionally to their contribution to the total dinitrogen pool, not by their occurrence in different locations within soil microsites. Unfortunately, when using ^{15}N to study nitrogen fixation, it must be remembered that some discrimination favoring ^{15}N atoms by nitrogenase in leguminous root nodules has been shown (Burris 1991). Furthermore, it is not possible to distribute evenly an externally supplied ^{15}N labeled mixture within the heterogeneous, complex soil structure. Even with these difficulties, ^{15}N is commonly used as a tracer for nitrogen in nitrogen fixation studies.

One means of counteracting the isotope discrimination problem is to use ^{15}N-depleted dinitrogen. For this method, the reduction in the atom percent ^{15}N in the ammonium present in the sample is measured in samples incubated in an

atmosphere containing dinitrogen from which the ^{15}N has been removed or highly reduced.

Other difficulties associated with large-scale utilization of ^{15}N to quantify nitrogen fixation in environmental samples are the requirement for a mass spectrometer to quantify enrichment or depletion of the ^{15}N in the ammonium pool extracted from the test system, the cost involved with purchase of the isotopically labeled substances, and the rather long incubation times in gas-tight systems required to insure that the labeled dinitrogen sources are not diluted by atmospheric sources.

Acetylene reduction: To overcome the methodological limitations involved with utilization of ^{15}N-labeled dinitrogen, a surrogate compound, reduced by nitrogenase at rates comparable to those associated with dinitrogen reduction, could be useful. Ideally, this substance should be quantified easily by methods readily available in most laboratories, and reasonably inexpensive. Fortunately, nitrogenase is not totally specific for dinitrogen as a substrate. This enzyme can also reduce nitrous oxide, cyanide, methyl isocyanide, azide, acetylene, and cyclopropene (Figure 12.2). The reduction of these compounds can serve as a measure of nitrogenase. The most useful alternate substrate for nitrogenase is acetylene in that it is not normally present in atmospheric samples, is relatively inexpensive, and can be quantified using gas chromatographs.

Since most studies of nitrogen fixation have an objective of assessing or predicting the quantities of dinitrogen that could be reduced in the samples, it would be useful if the quantities of ethylene produced from acetylene could be extrapolated to nitrogen fixation potential. The stoichiometry of the reduction of dinitrogen or acetylene by nitrogenase is as follows:

$$N_2 + 8H^+ \rightarrow 2NH_3 + H_2$$

$$3C_2H_2 + 6H^+ \rightarrow 3C_2H_4$$

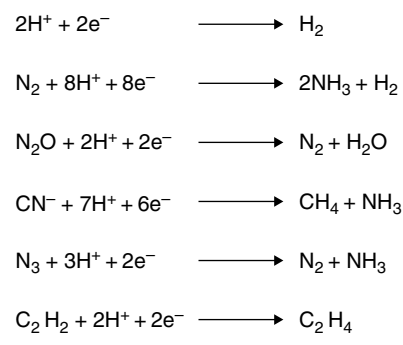

Figure 12.2 Some alternate substrates reduced by the nitrogenase system.

Considering the hydrogen ions incorporated into the acetylene and dinitrogen by nitrogenase, reduction of three acetylene atoms to three ethylene atoms should be equivalent to reduction of one dinitrogen to two ammonia atoms; that is, production of three ethylene molecules should represent the reduction of one dinitrogen.

Unfortunately, extrapolation of data collected from analysis of acetylene to estimate field nitrogen fixation rates is more complicated than this stoichiometric comparison predicts. Comparison of data derived from the assessment of nitrogen fixation in parallel samples using ^{15}N-labeled dinitrogen and acetylene suggests that this ratio of 3 : 1 (ethylene to dinitrogen) can be attained at times in culture and soil, but more commonly the ratio ranges from 0.75 to 4.5 or greater. Therefore, caution is necessary in interpretation of the acetylene-derived data. Due to this data inconsistency, nitrogen fixation potential measured by the acetylene procedure is generally designated "nitrogen fixation (acetylene)" so that readers may realize that the results are derived from extrapolation from an acetylene-based assay rather than from an assessment of dinitrogen reduction directly.

This discrepancy between predicted equivalence of acetylene and dinitrogen reduction from basic chemical principles and actual field results can be explained to a large degree by the different solubilities of dinitrogen and acetylene in water. Dinitrogen is poorly soluble in water. Thus, nitrogenase is rarely saturated by its primary substrate. In contrast, acetylene is highly water soluble. An acetylene concentration of 0.2 atm is usually sufficient to saturate nitrogenase. Therefore, when acetylene is used as a substrate for nitrogenase, all nitrogenase enzyme molecules in the sample are measured. (Since the substrate is at saturating concentrations in the reaction mixture, the reaction rate is proportional to the quantities of enzyme present.) When the nonsaturating concentrations of dinitrogen are used to assess nitrogen-fixing capacity, some of the enzyme is inactive and therefore undetected. (In the latter situation, the reaction rate is substrate-, not enzyme-concentration limited.)

A further complication in using the acetylene reduction procedure to estimate biological nitrogen fixation rates in field samples can result from reduction of the ethylene produced by nitrogenase. To accurately quantify nitrogenase activity, all the ethylene produced from reduction of acetylene must be detected. In some soil ecosystems, the ethylene produced by nitrogenase is oxidized by soil microorganisms. Some soil microbes are capable of using ethylene as a carbon and energy source. Thus, to assure that the ethylene detected during measurements of nitrogenase activity represents the total amount produced by nitrogenase, a control sample for disappearance of ethylene must be included in the study.

12.2 General Properties of Soil Diazotrophs

Nitrogen fixation is catalyzed solely by bacteria either living among the general soil microbial population or in symbiotic associations (i.e. *Rhizobium*–legume or actinorhizal symbioses or with fungi [lichens]). Rhizosphere-associated nitrogen-fixing bacteria (e.g. *Azospirillum* sp. growing in and around the root) can also be defined as existing in a loosely symbiotic relationship with the plant. Nitrogen-fixing microorganisms, that is *diazotrophs*, constitute a large group of marginally related bacteria. In contrast to the situation with nitrifying bacteria where the capacity to nitrify is sufficiently definitive that it can be used as a taxonomic trait, the extreme physiological and structural diversity of diazotrophs preclude using a single metabolic property, synthesis of nitrogenase, as a generic defining trait. Grouping nitrogen-fixing organisms into one or a few bacterial genera based on their capacity to reduce dinitrogen to ammonium is counterproductive. These organisms are found in such diverse groups as eubacteria, the photosynthetic cyanobacteria (formerly known as the blue-green algae), and actinomyctes.

The only clear commonalties between the diazotrophs is their general classification as bacteria and their ability to fix nitrogen. Diazotrophs are aerobes, facultative anaerobes, or anaerobes. They may derive their energy from organic substances, inorganic compounds, or directly from solar energy. Diazotrophs grow as single cells or chains of individual cells (e.g. rhizobia, clostridia), as loosely associated colonies (e.g. some blue-green algae), and as mycelial structures (e.g. the actinomycetes). This metabolic and physical variability enables these organisms to contribute to fixed nitrogen pools in essentially any ecosystem where microbes are capable of growth and reproduction (see Table 12.1).

12.2.1 Free-Living Diazotrophs

From a consideration of the wide variety of ecosystems in which free-living diazotrophs are found, it can be concluded that a vast diversity of diazotrophs must exist. Indeed, bacteria capable of fixing nitrogen while growing independently in soil are distributed among at least 26 genera, 11 families, and three orders of bacteria. It must be noted that within each individual genus, the number of species capable of fixing nitrogen is small, and that not every strain of a diazotrophic species will necessarily be capable of fixing nitrogen.

To provide an indication of the limitations to growth and their contributions to total fixed nitrogen in soil ecosystems, representative examples of aerobic, anaerobic, and autotrophic nitrogen-fixing bacteria will be discussed. The relationship of some of these organism to the properties of their natural habitats is analyzed in the next section. The individual species examined are not necessarily the most important in

Table 12.1 Examples of diazotrophs commonly found in soil, rhizosphere, and soil-linked aquatic systems

Habitat and metabolic grouping	Microbial groups
Nonassociated, aerobes, heterotrophs	*Azotobacter* spp. *Bacillus* spp. *Beijerinckia* spp. *Derxia* spp.
Nonassociated, aerobes, lithotrophs	*Thiobacillus* spp.
Nonassociated, anaerobes, heterotrophs	*Clostridium* spp. *Desulfovibrio* spp.
Aquatic (swamps, bogs, rice fields), aerobes, phototrophs	Cyanobacteria (blue-green algae)
Aquatic (swamps, bogs, rice fields), anaerobes, phototrophs	*Chaetomium* spp. *Rhodopseudomonas* spp. *Thiospirillum* spp.
Rhizosphere (loosely associated), facultative aerobes, heterotrophs	*Enterobacter* spp. *Erwinia* spp. *Klebsiella* spp.
Rhizosphere (loosely associated), aerobes, heterotrophs	*Azospirillum* spp. *Pseudomonas* spp.
Root nodules (legumes or angiosperms)	*Frankia* spp. *Rhizobium* spp.

any given ecosystem or in soil ecosystems in general. In many cases, they simply represent the more commonly studied. Quite commonly, the frequency of isolation and evaluation of individual diazotrophs is determined by the ease of isolation and their propensity for reproducible growth of the organism in axenic culture rather than the proportion of the soil nitrogen-fixing population that they represent.

Aerobic, free-living diazotrophs: The three most studied free-living, strict aerobes are *Azotobacter chroococcum*, *A. beijerinckia* (*Beijerinkia* sp.), and *Derxia gumosa*. From the number of reports in the literature, the conclusion could easily be reached that *A. chroococcum* is the most widespread species in neutral or alkaline soils. This conclusion is tentative at best and may easily be in error. The frequency with which this organism is studied may relate to the fact that it has been commonly studied in general bacteriology classes. Thus, soil microbiologists are well versed in the methods for isolating and growing this bacterial species.

Azotobacter chroococcum is a strict aerobe with a temperature optimum of about 30 °C. The numbers of these organisms in soil range from undetectable to several

thousand g^{-1} soil. It is unusual to find large populations of this organism in soil, so it is unlikely to be an important contributor to total ecosystem fixed nitrogen inputs, except in some specialized situations (e.g. soils receiving inputs of fixed carbon substances that can provide energy for growth of the *Azotobacter* spp.). *A. beijerinckia* is dominant in acidic soils – pH values as low as 3.0. These organisms are common in tropical soils, rarely being found in temperate soils. They have also been reported to occur in high Arctic soils (Jordan and McNicol 1978) whereas both *A. chroococcum* and *A. beijerinckia* populations have been detected in Antarctic soils (Vishniac 1993). Similarly, *D. gummosa* is common in tropical soils of South America with pH values ranging from about 4.5 to 6.5.

A variety of nonsymbiotic diazotrophs are frequently found in rhizosphere soils. These organisms include *Azospirillum* species plus a variety of enteric bacterial species. *Azospirillum lipoferum*, which was originally described by Beijerink in 1922 and named *Spirillum lipoferum*, forms loose symbiotic associations on grasses. These spiral-shaped gram-negative organisms typically grow around roots and also penetrate the roots to grow intercellularly. These organisms use root exudates for their carbon and energy source while fixing nitrogen. The latter group of nitrogen-fixing bacteria – the enterics – includes *Klebsiella* sp.

Anaerobes and facultative anaerobic bacteria: The most commonly encountered anaerobic diazotrophs are *Clostridium* spp. These organisms are nearly universally present in poorly drained soils as well as in anaerobic microsites of arable soils. *Klebsiella* spp. are the most commonly observed facultative anaerobic nitrogen-fixing bacteria. These organisms are widely distributed in aquatic and terrestrial environments, especially in rhizosphere soils. In arable lands, clostridial populations commonly range from 10^2 to 10^5 propagules g^{-1} soil although under optimal conditions, populations as high as $10^6\,g^{-1}$ have been detected. In contrast, *Klebsiella* spp. have been found in rhizosphere samples at population densities of 10^6–$10^8\,g^{-1}$ root tissue (Evans et al. 1972). The nitrogen fixation efficiency of these organisms is comparable to that reported for aerobic organisms (2–20 mg N fixed g^{-1} carbohydrate consumed). It has been suggested that in some systems, such as forest soils, nitrogen fixation is probably limited to anoxic microsites (e.g. Limmer and Drake 1996).

Cyanobacteria: Soil microbiologists tend to disregard the input of algae to soil biogeochemical cycles, but blue-green algae can provide significant inputs of fixed nitrogen into land-based ecosystems. These photosynthetic organisms are found primarily in surface soil crusts. Significant algal populations may develop on soil surfaces, especially in the presence of a thin layer of free-standing water. Approximately one-tenth of the established genera of cyanobacteria contain diazotrophs. The absolute contribution of these organisms to soil nitrogen economy is difficult to assess since their distribution is frequently localized, but significant contributions have been measured in rice field soils.

Due to the photosynthetic nature of these organisms, the quantities of nitrogen fixed are independent of the availability of an external chemical energy supply. Yields of fixed nitrogen due to cyanobacteria can be highly meaningful. In rice paddy soils, contributions up to 30 kg N ha^{-1} yr^{-1} are commonly reported. Rychert and Skujins (1974) found that algae and lichens in soil crusts of the Great Basin Desert could produce fixed nitrogen at rates up to 84 g N ha^{-1} yr^{-1} (acetylene) in the laboratory. The rates they observed would extrapolate to 10–100 kg N ha^{-1} yr^{-1} if comparable efficiency were achieved in field samples.

12.2.2 Examples of Function of Nonsymbiotic Diazotrophs in Soil Ecosystems

Although population densities of nonsymbiotic diazotrophs may suggest that they are minimal contributors to fixed nitrogen levels in most ecosystems, soil ecosystems wherein they are major fixed nitrogen producers are easily recalled. They are meaningful players in nitrogen cycling in such ecosystems as wetlands (natural and agricultural managed sites) as well as plant rhizospheres. Selection and function of diazotrophs for function in these two types of ecosystem are particularly instructional in that conditions in each of these system types exemplify extremes in soil properties conducive to the function of nitrogenase. Both wetland soils and rhizosphere sites can contain elevated concentrations of readily metabolizable organic matter in comparison to generally arable soils, and oxygen tensions are reduced in both sites with increased probability of development of anaerobic microsites.

12.2.3 Diazotrophs in Rhizosphere Populations

The rhizosphere is home to a variety of heterotrophic diazotrophs exemplified by members of the family Enterobacteriaceae (including *Enterobacter agglomerans*, *K. pneumoniae, Enterobacter cloacae*, and *Erwinia herbicola* [e.g. Haahtela et al. 1981; Lindberg and Granhall 1984; Pedersen et al. 1978]), *Bacillus* species (e.g. Lindberg and Granhall 1984), and *A. lipoferum* (e.g. Haahtela et al. 1981). The presence of the enterobacteria and *Azospirillum* species conforms to *a priori* notions regarding this ecosystem; that is, the organisms are heterotrophs and capable of functioning under reduced oxygen tensions or in the total absence of oxygen. Indeed, since both aerobic and anaerobic microsites occur in the vicinity of the growing root, even the isolation of strict aerobes, such as the example of a *Bacillus* species cited above, from this environment is not problematic.

A perhaps more significant concern for a student of the environment relates to the significance of the contribution by diazotrophs residing in the rhizosphere to the fixed nitrogen resources of the growing plant. Although situations have been demonstrated where a significant role of rhizosphere diazotrophs in provision of

plant-fixed nitrogen is suggested (Döbereiner 1997), the importance of free-living diazotrophs in the rhizosphere in plant nitrogen metabolism is more commonly described as minimal but significant. Acetylene and ^{15}N-labeled dinitrogen are reduced in the rhizosphere albeit at minimal levels. For example, Giller et al. (1988) found little to no nitrogen fixation in the rhizosphere of sorghum (*Sorghum bicolor*) and millet (*Pennisetum americanum*). With the sorghum, measurable nitrogen fixation was only detected on one occasion whereas with the millet, nitrogen fixed was less than 1% of the plant nitrogen accumulated. Berestetskii and Vasyuk (1983) found weak nitrogen fixation activity with spring wheat (*Triticum aestivum*), *Poa pratensis*, and *Phleum pratense*, but higher activity with millet. Pedersen et al. (1978) estimate that the maximum rate of nitrogen fixation (extrapolated from acetylene reduction data) in spring wheat and sorghum was 2.5 g N ha^{-1} d^{-1}.

Interestingly, although diazotrophs functioning in rhizosphere or rhizoplane ecosystems rely on root exudates to meet fixed carbon and energy requirements, these nitrogen fixation rates are within the range generally observed for free-living diazotrophs in less nutritionally luxuriant soil sites. This observation suggests that although *a priori* it could be anticipated that the rhizosphere-residing nitrogen-fixing bacteria should not be energy limited, the actual nitrogen fixation rates detected indicated that competition with the general rhizosphere microbial community for carbon and energy supplies limits the capacity of the diazotrophs to exploit nutrients in root exudates.

The contribution of rhizosphere nitrogen fixation to the nitrogen budget of higher plants is minimal (see Giller and Day 1985 for a further discussion of this topic). From the view of managing soil biologically fixed nitrogen to decrease anthropogenic intervention and increase system sustainability, the question emerges of whether the nitrogen-producing capacity of the rhizosphere ecosystem can be increase. Although optimal conditions for nitrogenase function can be anticipated to occur in the rhizosphere, either microsite conditions vary from these anticipated situations or the efficiency of nitrogen fixation by the diazotrophs in the field is not sufficient to lead to meaningful fixed nitrogen contributions to the plant.

Difficulties with heterogeneity of the microenvironment of the rhizosphere encountered by nitrogen-fixing bacteria are exemplified by evaluating the impact of variation of oxygen tension of the root environment on *in situ* nitrogen fixation. Haahtela et al. (1983) found that anaerobic conditions were required for maximal expression of nitrogenase activity of *Klebsiella* sp. and *Enterobacter* sp. isolated from plants. Similarly, in roots of corn (*Zea mays*), maximal acetylene reduction was noted at partial pressures of oxygen of 1–2 kPa. The activity was strongly inhibited with oxygen tensions above or equal to 6 kPa (Alexander et al. 1987). Alexander and Zuberer (1989) found that with ^{15}N-labeled dinitrogen ammonium production was 200-fold greater at 2 kPa molecular oxygen than at 10 kPa. Thus, it could be predicted that a controlling factor for nitrogen fixation in the rhizosphere

would be the development of anaerobic microsites. Total anaerobiosis in this habitat is clearly precluded by the requirement for oxygen for respiration of the root tissue.

Management of rhizosphere diazotroph populations: A potential means of reducing fertilizer expense for cropped (nonlegume) systems and anthropogenic intervention in reclamation projects is to optimize *in situ* inputs of biologically fixed nitrogen through manipulation of rhizosphere populations. Since habitats favorable to growth and development of diazotrophs exist around the plant root, the objective of such studies can be reduced to selection of efficient nitrogen-fixing strains of rhizosphere bacteria that are capable of competing with indigenous microbes when returned to the root ecosystem. An apparently optimistic expectation for achievement of such goals is possible in that selection of natural genetic variants and production of genetic engineered strains with improved nitrogen fixation efficiency can readily be accomplished in the laboratory. Furthermore, the potential exists for the manipulation of rhizosphere populations for the successful inoculation of these laboratory-cultivated strains into field situations. Application of these procedures is exemplified by studies of *Azospirillum* spp. which are normal components of the rhizosphere microbial community, fix nitrogen, and are amenable to laboratory culture.

As with any inoculation procedure, be it soil or rhizosphere inoculation, the initial concerns with amendment of axenically cultivated strains are (i) "Can the desired effect be observed in the ecosystem following addition of the test strain (e.g. stimulation of plant biomass synthesis?" and (ii) "Does the alteration of the ecosystem result from the anticipated activity of the amended microbe or is an alternative mechanism operative?" Once responses to these questions are acquired, then considerations regarding reproducibility, stability, and magnitude of the amendment response must be addressed.

Inoculation of a variety of grass varieties with *Azospirillum* strains does increase plant biomass production (e.g. Bashan and Holguin 1997; Desalamone et al. 1996; Gaskins et al. 1977; Smith et al. 1976; Subba-Rao et al. 1978). Several mechanisms may be proposed to explain this increase in plant growth by inoculation with cultures of *Azospirillum*. Explanations of the stimulation of biomass production include (i) augmented ammonium supplies produced by the *Azospirillum* sp. (i.e. nitrogen fixation), (ii) plant hormone synthesis by the bacterium (e.g. Tien et al. 1979), (iii) alteration of root structure (e.g. Fallik et al. 1988; Hadas and Okon 1987; Jain and Patriquin 1984), and (iv) response to increased plant nutrients provided by mineralization of dead *Azospirillum* cells. (See Vanderbroek and Vanderleyden 1995 for a review of the genetics of phytohormone production, nitrogen fixation, and mechanisms of plant root attachment.)

It is probable that all these mechanisms function in part in regard to *Azospirillum* inoculation of grasses since nitrogenase synthesis and activity appear not to be the

primary explanation for increases in plant productivity. Estimates of fixed nitrogen produced by *Azospirillum* sp. in the rhizosphere range from $2\,kg\,N\,ha^{-1}$ (von Bülow and Döbereiner 1975) to as much as $90\,kg\,N\,ha^{-1}$ (Döbereiner et al. 1972, 1973) with inoculated grasses in South America. Other studies have reported low levels of nitrogen fixation in temperate grasses (e.g. Barber et al. 1976; Harris et al. 1989; Okon et al. 1983). Alternatively, some impact of plant hormone production by the *Azospirillum* sp. used as an inoculant is logical. The bacterially produced plant hormones would stimulate root biomass production (e.g. Tien et al. 1979). Bashan and Dubrovsky (1996) have shown mixed results of inoculation with *Azospirillum* spp. on shoot to root ratio. In about half of the cases, this ratio was increased whereas in the remainder the ratio was decreased. Cation accumulation by the plant may (Lin et al. 1983) or may not (Bashan et al. 1990) be meaningful.

These data suggest limitations in yields of fixed nitrogen in inoculated rhizospheres. As our understanding of the genetics mechanisms associated with nitrogen fixation in *Azospirillum* and with ability of the bacterium to compete with indigenous rhizosphere populations improves, the probability of developing an economic and effective rhizosphere inoculation procedure will increase. Also, along with using genetic modification to improve competitive ability of *Azospirillum* cells introduced into the rhizosphere, fungicide or bacteriocides may be added with the diazotroph to increase the probability of establishment of the culture (Bashan 1986a). With this procedure, the inhibitors are used to limit the activity of indigenous populations until the foreign bacterial strain can become established, thereby giving the amended organism a competitive advantage.

Associated with selection of appropriate strains for inoculation of root or seed tissue is the development of an effective inoculation method. Viable cells must be delivered to the appropriate habitat on the root at a time of root development when maximal benefits to the plant are accrued and the conditions in the microsite wherein the microbes are anticipated to function are optimal for nitrogen fixation. *Azotobacter* populations can become established on grass roots through inoculation of either seeds or root tissue, although seed inoculation appears to be the most effective means of delivery (Bashan 1986b). One advantage of seed inoculation is that the amended bacterial strain is present as the nascent root tissue is produced. Thus, instead of having to compete with established bacterial populations, as would be the situation when roots are inoculated directly, the competition is reduced to a race between seed and soil flora to colonize the newly emergent root tissue.

Should the objective be to inoculate root tissue directly, the ability of the added cells or their progeny to spread from inoculated to noninoculated roots becomes important. Roots of infected plants can serve as a vector for transmission of the exogenous bacterial strain to noninfected populations (Bashan and Levanony 1987, 1989). In fact, weeds growing between rows of crop plants can serve to transport the added bacterial strain (Bashan and Levanony 1989). Thus, although a

time delay in spread of the inoculant must occur with direct inoculation of plant roots in the field, diffusion of inoculated bacterial strains throughout a field can be anticipated to result eventually. For this process, active bacterial cultures may be added to the soil surface adjacent to the plant stem and washed into the rhizosphere, plant roots could be dipped into the inoculum prior to planting, or the inoculum could be banded adjacent to the plant in a region where the roots of the growing plant would be anticipated to penetrate. As has been shown with rhizobial inoculation of legumes (see Chapter 13), a variety of carriers may be used for introduction of *Azospirillum* inoculum into soil (e.g. Bashan 1986c). Each of these techniques would be less efficient than seed inoculation (i.e. from a time as well as quantity of inoculum which would have to be used), but with highly competitive and nitrogen fixation-efficient diazotroph strains, such procedures may become more feasible.

An interesting variation of the observed effect of the inoculation of nonlegumes with *Azospirillum* spp. is the situation where legumes are inoculated with a mixture of the appropriate rhizobial strain for the legume plus *Azospirillum* sp. (e.g. Bashan and Holguin 1997; Burdman et al. 1997; Galal 1997). With appropriate ratios of the two microbial strains, nodulation and nitrogen fixation of the association are increased over those seen with plants inoculated with the rhizobial strain alone.

12.2.4 Dizaotrophs in Flooded Ecosystems

Wetland soils are sites of limited but active biological nitrogen fixation. The quantities of fixed nitrogen gained by this source are within the range commonly reported to be produced by free-living microbes in soils in general. For example, Waugman and Bellamy (1980) estimate nitrogen fixation inputs due to heterotrophic bacteria in German mires (using the acetylene reduction method) to range from 0.07 to 2.1 $g\,N\,m^{-1}\,yr^{-1}$. Slightly higher fixed nitrogen contributions have been reported for Gulf coast salt marshes (USA) at 4.5 and 15 $g\,N\,m^{-2}\,yr^{-2}$ (Casselman et al. 1981). Since biomass productivity is low in swampy environments due to the flooded conditions, biologically fixed nitrogen can be a major source of this essential plant nutrient.

Free-living bacteria are responsible for nitrogen fixation in swampy soils. As might be anticipated considering the oxygen-limiting conditions of marshes, anaerobic bacteria are major participants in this process. Dicker and Smith (1980) found that *Clostridium* sp. and *Desulfovibrio* sp. accounted for a major portion of the nitrogen-fixing activity in a Delaware salt marsh, although large populations of *Azotobacter* sp. were also present in the samples. Co-occurrence of obligate aerobes and anaerobes in this ecosystem appears to be contradictory, but consideration of the heterogeneity of the soil profile (i.e. water-saturated soil overlain by

flowing water) reveals that it is reasonable to anticipate occurrence of both aerobic and anoxic microsites. Oxygen-bearing water flowing into the system provides for the sustenance of aerobic microsites whereas activity of the aerobic bacteria in the system creates anaerobic sites. Furthermore, sequestering of water into nonflowing situations within the soil profile also results in development of anaerobic habitats within the soil profile (stagnation).

The diversity of diazotrophs present in swampy soils indicates the existence of a variety of energy sources supportive of microbial growth. Metabolic energy supply in these ecosystems is provided by (i) root exudates (e.g. from the *Spartina alterniflora* Loisel. plants in the salt marshes), (ii) accumulations of partially decomposed organic matter under anoxic conditions (i.e. products of anaerobic decomposition of biomass substituents), and (iii) solar energy (e.g. growth of cyanobacteria in the aquatic portion of the site).

Rice paddies provide an excellent example of the function of these various diazotroph groups in a managed, flooded ecosystem. A diverse population of autotrophic bacteria is found in the flood waters (e.g. Habte and Alexander 1980a; Kulasooriya and de Silva 1981). For example, 73 strains of cyanobacteria distributed taxonomically among 21 genera were isolated from rice soils in central Sri Lanka (Kulasooriya and de Silva 1981). Similarly, the rhizosphere contains an active heterotrophic diazotroph population (e.g. Habte and Alexander 1980b; Yoo et al. 1986). It should also be noted that these heterotrophic strains may be active on aboveground plant surfaces (Ito et al. 1980). The activities of the latter populations were shown to be approximately 2.5-fold more efficient at fixing nitrogen than rhizosphere populations (Ito et al. 1980).

Wetland systems, with their low but significant biologically fixed nitrogen inputs, provide an even more vivid picture of the complexity of diazotrophic communities within a single ecosystem than is provided by the rhizosphere examples discussed above. This diversity in physiological ability provides a genotypic foundation for long-term sustenance of the system under ever-changing conditions. Balance between fixed nitrogen inputs from heterotrophic or autotrophic populations, aerobes or anaerobes, rhizosphere or leaf sheath populations is dependent upon such site properties as water influx rate, depth of flooding (i.e. drained to submerged), diurnal variation of temperature, and light influx.

12.3 Conclusions

The capacity to fix nitrogen is widely distributed among the bacterial genera, although only a limited number of species are actually capable of catalyzing the process. Nitrogenase, the enzyme responsible, is highly conserved among this

diverse group of bacteria. Two characteristics of this reductive process dictate the yield of fixed nitrogen in various terrestrial environments. First, nitrogen fixation is an energy-intensive process. Thus, large quantities of dinitrogen are fixed only in situations where there is a high energy supply (e.g. symbiotic associations involving formation of root nodules and through action of photosynthetic bacteria). Diazotrophs growing in competition with the general heterotrophic soil microbial community contribute small quantities of fixed nitrogen to the biological community. Second, nitrogenase is an oxygen-labile enzyme so each diazotroph must expend resources to maintain anoxic conditions in the environment of active nitrogen fixation. These two overall traits of the nitrogen fixation process and the diverse group of bacteria capable of catalyzing the reaction combined with the extremely heterogeneous soil ecosystem select for a highly diverse indigenous population of diazotrophs in any terrestrial ecosystem. For example, the rhizosphere can contain aerobic, facultative anaerobic and strictly anaerobic diazotrophs when the oxygen sensitivity of the organisms alone is considered. This diversity of *in situ* populations and the variations in phenotypic expression of nitrogen-fixing efficiency allow cautious optimism for genetic manipulation and selection of diazotrophs that can be introduced into soil systems to improve contributions to soil fixed nitrogen resources.

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13

Biological Nitrogen Fixation

Root nodule-based nitrogen fixation associations are exemplified by *Rhizobium*–legume and actinorhizal symbioses. As noted in Chapter 12, these interactions between diazotrophic bacteria and plants are major biological contributors of fixed nitrogen in soil-based ecosystems. The predominance of this fixed nitrogen source results from (i) the agricultural exploitation of *Rhizobium*–legume symbioses and (ii) the advantages conferred on these symbiotic diazotrophs by the ready availability of plant-produced photosynthate. Recall that nitrogen fixation is an energy-intensive process and the heterotrophic diazotrophs living in the legume or actinorhizal nodule have more energy available to devote to nitrogen fixation than do diazotrophs living nonassociated or loosely linked with plants.

Actinorhizal associations are interactions of nonleguminous angiosperms with actinomycetes of the genus *Frankia*. *Rhizobium*–legume symbioses are the most studied, to a large degree perhaps due to their agricultural and economic importance. From a total ecosystem viewpoint, actinorhizal symbioses are also major contributors to terrestrial nitrogen cycling. The latter symbioses are more characteristic of nonmanaged ecosystems. Commonly considered actinorhizal associations are bayberry (*Myrica pensylvanica*) and *Casuarina*, a nonleguminous tree found in tropical and semiarid countries, plus a variety of ferns. Actinorhizal associations are frequently found in acidic or physically or chemically stressed soils. Nitrogen-fixing legumes are more common in agricultural soils and other ecosystems with soils of pH 5 or greater. Additionally, the ability of actinorhizal associations to function in acid-stressed sites suggests a potential for their use in acid-impacted reclamation sites.

The primary objectives for this portion of our analysis of the symbiotic diazotrophs are to elucidate the properties of the bacteria and the host plant that control the efficacy and predominance of the associations in ecosystems and to use these interactions as models for evaluation of their potential for manipulation for management of biogeochemical cycles in soil ecosystems. Topics were selected for

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analysis to stress the environmental aspects of nitrogen fixation in nodulated plant root systems. Biochemical and biological aspects of the topic will be analyzed only where needed to provide a foundation for understanding their impact on the occurrence of nitrogen fixation by these symbiotic associations in native ecosystems. For a more detailed discussion of the biochemistry or biology of actinorhizal or *Rhizobium*–legume interactions, refer to essentially any basic general microbiology or biochemistry text. Several reviews that provide an overview of these topics in greater detail than can be provided herein are also presented.

13.1 Rhizobium–Legume Symbioses

Due to the extensive cultivation of legumes, the greatest documented contribution of fixed nitrogen into land-based systems results from the infection of legume roots by species of the bacterial genera *Rhizobium* and *Bradyrhizobium*. A tremendous potential for contribution of fixed nitrogen to soil ecosystems exists among the legumes. There are approximately 700 genera and about 13 000 species of legumes, only a portion of which have been examined for the ability to fix nitrogen. Estimates are that the rhizobial symbiosis with the somewhat greater than 100 agriculturally important legumes contributes nearly half of the annual quantity of biological fixed nitrogen entering soil ecosystems.

The bacterial symbionts are gram-negative, nonspore-forming, aerobic rods. Some strains are nonmotile, but typically rhizobia are motile. Rhizobia metabolize several carbohydrates, possibly with the production of acid, but no gas. The symbiotic association with legumes is a distinguishing classification trait of the genus (rhizobial species are grouped into the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* [stem nodulating organisms, e.g. Adebayo et al. 1989]). Speciation of these bacteria is traditionally based on host specificity as defined by cross-inoculation grouping. Strain-specific DNA probes are proving useful in studying the occurrence of these organisms in soil (e.g. Watson et al. 1995) as well as in studying their taxonomy and phylogeny (e.g. Agius et al. 1997; Anyango et al. 1995; Young and Haukka 1996). For example, sequencing of small subunit ribosomal RNA supports division of rhizobia into three genera (Young and Haukka 1996). Serological typing, DNA analysis, and legume infection have also been used to detect, identify, and quantify rhizobia. Particularly reassuring is the fact that the groupings of rhizobia delineated by the three types of analyses generally agree.

13.1.1 Grouping of Rhizobial Strains

From the view of a microbiologist, rhizobia constitute a somewhat nondescript group of bacteria. Classification schemes based on data derived from traditional physiological tests are inadequate to separate the genus into meaningful groupings.

An alternative trait common to rhizobia that provides a reproducible method for their classification that was testable by methods available to early microbiologists is host specificity. The resultant association of strains into groups based on host specificity is called *cross-inoculation groups*. Cross-inoculation groups are, by definition, a collection of leguminous species that develop nodules when exposed to bacteria obtained from the nodules of any member of that group. More than 20 cross-inoculation groups have been established. Of the seven most studied groups, six are sufficiently described to designate the responsible bacteria as species. The most studied cross-inoculation groups are as follows.

- *The alfalfa group*: alfalfa (*Medicago* spp.) and sweet clover (*Melilotus* spp.) nodulated by *Rhizobium meliloti*.
- *The clover group*: clovers (*Trifolium* spp.) nodulated by *Rhizobium trifolii*.
- *The pea group*: pea (*Pisum* spp.) and vetch (*Vicia*) nodulated by *Rhizobium leguminosarum*.
- *The bean group*: beans (*Phaseolus* spp.) nodulated by *Rhizobium phaseoli*.
- *The soybean group*: soybeans (*Glycine* spp.) nodulated by *Bradyrhizobium japonicum*. *B. japonicum* and *Bradyrhizobium* spp. (symbionts of the cowpea group) were previously classified as slow-growing variants of *Rhizobium japonicum*.
- *The cowpea group*: this group has not achieved a species designation, but these organisms nodulate a variety of legumes including cowpeas (*Vigna* sp.), kudzu (*Pueraria* sp.), peanuts (*Arachis* sp.), and lima beans (*Phaseolus* sp.).

The cross-inoculation system has provided a useful classification system for rhizobial strains, but some major problems have historically been associated with its application. Some of these are logistical. For example, considerable greenhouse space is necessary to grow the legume seedlings required for accurate determination of the identity of the *Rhizobium* strain of interest. Of perhaps greater concern in using this method to group newly isolated rhizobial strains is the potential for some organisms to nodulate more than one legume species. For example, soybean and cowpea groups contain strains of rhizobia isolated on legume groups that nodulate other legume species. This variation in nodulation specificity is termed *symbiotic promiscuity*.

Thus, the validity of the cross-inoculation system has been challenged in some situations. The procedure works reasonably well for the frequently studied rhizobia nodulating common, agricultural crops of temperate regions. More difficulties may be encountered as a greater proportion of the currently little studied rhizobia that nodulate tropical legumes are classified. Fortunately for the classification system, many rhizobia do nodulate legumes outside their particular class, but these nodules are generally nonfunctional in nitrogen fixation (i.e. they are not effective). Thus, the designation of species or group based on formation of effective nodules is sustained. It should be noted that not all of the literature reports of symbiotic promiscuity represent true diversity in legume species specificity.

In some cases, apparent host diversity has been the result of contamination of the rhizobial cultural with other rhizobial strains (Leps et al. 1980).

Cross-inoculation groups have provided a reasonably stable philosophical basis for the taxonomic scheme for grouping rhizobial strains. But the extent of the species isolated was/is greatly limited – basically to a few rhizobial strains. This myopia resulted at least in part from the fact that the crops and soils examined tended to be limited to those associated with major agricultural food crops. A variety of methods, including genetic analysis and nutritional traits, are now commonly studied in both agricultural and "wild" soils. These techniques plus examination of a more diverse grouping of soils than previously used in the majority of biological nitrogen fixation have reinforced the concepts of species designations derived by more classical methods (e.g. Brunel et al. 1996; Madrzak et al. 1995; Texfaye et al. 1997), revealed strain variations within individual species (e.g. Dye et al. 1995; Hernandezlucas et al. 1995; Urtz and Elkan 1996; Vanberkum et al. 1995), and detected occurrence of specific strains in soils and nodules (e.g. Desa et al. 1997; Hartmann and Amarger 1991; Sessitsch et al. 1997; Simon et al. 1996). As the nuances of the implications of the application of these procedures to the study of rhizobial taxonomy become better understood, a more inclusive grouping of microbial strains will emerge. (See Elkan and Bunn 1992 for examples of the status of rhizobial taxonomy.) The utility of study of more general, less specific soil sources is demonstrated by work of de Meyer et al. (2015) and Howieson et al. (2013). Examples of DNA sequence and gene diversity utility are provided by de Meyer et al. (2011), Bellenger et al. (2014), Izquierdo and Nüsslein (2006), Magadlela et al. (2017), and van Cauwenberghe et al. 2015.

13.1.2 Rhizobial Contributions to Nitrogen Fixation

Rhizobia are capable of fixing nitrogen in axenic culture. Pagan et al. (1975) developed a defined medium and culture procedure that allows nitrogen fixation by free-living rhizobial strains. With this method, the bacteria are grown to a high density on yeast extract and mannitol agar, suspended in sterile water and spread on a defined agar medium. Other studies have shown that nitrogenase activity of *R. japonicum* growing in defined medium is dependent upon the carbon source metabolized (Kurz and Larue 1975) and oxygen tension of the growth medium (Tjepkema and Evans 1975). Microaerophilic conditions are required for induction of nitrogen-fixing activity by the rhizobial cells. Kiester and Evans (1976) found that the optimal oxygen level for nitrogen fixation by *R. japonicum* and a *Rhizobium* sp. was approximately 0.1% in the gas phase. Carbon dioxide is also required by the rhizobial cells. Aguilar and Favelukes (1982) found an obligatory need for carbon dioxide for nitrogen fixation under microaerophilic conditions. This effect of carbon dioxide is mediated through ribulose bisphosphate carboxylase activity

(Manian et al. 1984). Once the environmental conditions are optimized, the rate of nitrogen fixation by free-living cells is comparable to that observed with bacteroides isolated from soybean nodules.

13.1.3 Nodulation of Legumes

Rhizobia in soil are capable of infecting roots of susceptible plants to produce root nodules (Figure 13.1). These nodules have a structure characteristic for the various symbiotic associations. Both the plant and the infecting bacteria control the development of the nodule structure. This summary of the process of formation

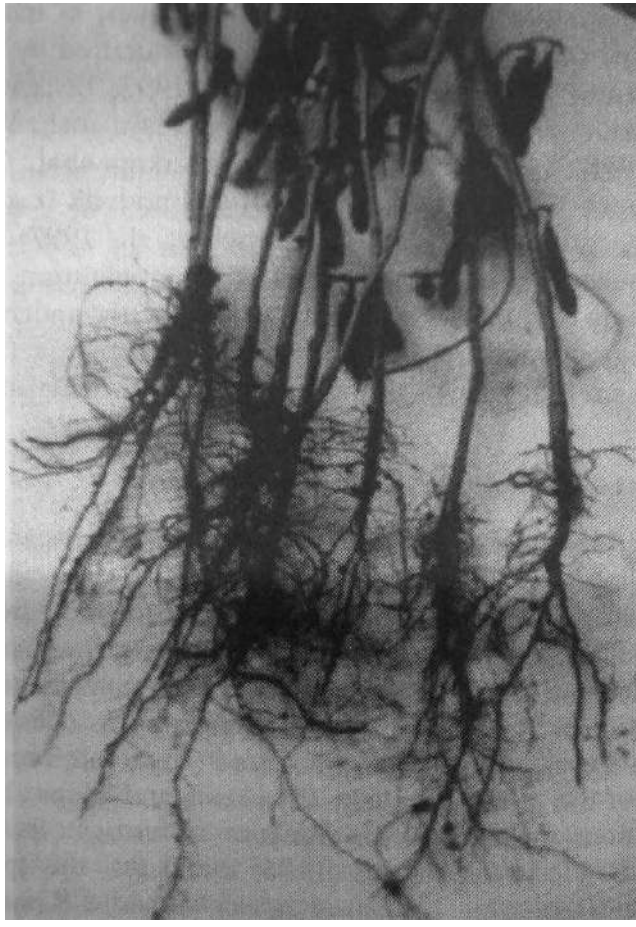

Figure 13.1 Root nodules formed by *Bradyrhizobium japonicum* and soybeans.

of legume root nodules can be divided into three topic areas: (i) properties of the plant and microbe facilitating contact and binding of rhizobial cells to the host root, (ii) recognition of susceptible host roots and attachment, and (iii) root invasion processes and development of effective nodules. A variety of excellent reviews describing the biological, genetic, and biochemical aspects of nodule formation have been published (e.g. Brewin 1991; Caetano-Anollés and Gresshoff 1991). Thus, only the salient details of the process will be outlined here. Selected primary research publications are cited to exemplify studies of the interaction of rhizobial strains and their hosts.

Encounter between susceptible roots and rhizobia: Although their population densities may be low, rhizobia are found in most soils, even those sites where legumes have not been cultivated for many decades. Therefore, limitations in frequency of nodule formation in native ecosystems tend to result more from the rate of encounter of infecting bacteria and host rather than presence of the symbiotic partners. Considering the heterogeneity of soil, the size of the bacterial cell, and the low proportion of the A horizon of the soil profile occupied by plant roots, random encounter between stationary bacterial cells and growing roots must be considered to be an inefficient means of inducing nodulation. Fortunately, a variety of basic properties of the soil system enhance this naturally low probability. First, in undisturbed soils, rhizobial cells are concentrated in the vicinity of the decaying root from previous growing seasons. The probability of infection of nascent root tissue is therefore enhanced by the propensity of newly developing root systems to grow into preexisting root channels.

A further increase in nodulation frequency could result from the movement or migration of infective bacteria which occurs within the soil profile. This migration of the rhizobial cells is primarily passive. That is, it usually does not involve directed motility of the cells (Issa et al. 1993a, b). The bacteria are carried by water flowing through soil macropores. Macropores are large passages in the soil matrix that allow the ready movement of soil air and water. These pores include the spaces between soil aggregates as well as the channels created by growth of roots (root channels) and movement of animals (e.g. earthworm channels). Passive transport of soil bacteria with leaching water provides for dispersion of cells over relatively large distances (in the A horizon, millimeter to centimeter distances). Note that this predominance of passive transport does not imply that directed motility of bacteria plays no role in nodulation. Compared to the situation with passive transport of cells, active motility of individual bacterial cells is of greater importance in movement of the cells in the microsite in response to exudate production by the growing root (chemotaxis; see below).

This differentiation of impact between passive and active aspects of bacterial mobility in soil does not preclude conference of a competitive advantage to rhizobial cells by the capacity for active motility. It must be noted that a number of

reports exist in the literature supporting as well as opposing the conclusion that motile strains of rhizobia have an enhanced potential to occupy legume nodules compared to nonmotile bacteria. The seemingly contradictory conclusions result to a large degree from variation in the properties of the laboratory- or greenhouse-incubated soils used in the experiments as well as from the various population densities of bacteria used in the experiments. It is indisputable that motility of rhizobia is not required for nodule formation yet a number of studies have shown both enhanced occurrence of nodulation by mobile strains compared to comparable nonmotile strains (e.g. Gulash et al. 1984; Hunter and Fahring 1980; Malek 1992; Napoli and Albersheim 1980) and no benefit for motility (Ames et al. 1980; Issa et al. 1993a, b; Liu et al. 1989).

Many of the contradictory results are attributable to variation in the importance of chemotaxis of infective rhizobia under the various experimental conditions. In situations where both motile and nonmotile strains have equal access to susceptible root tissue, no benefit is gained from mobility. In contrast, if the soil moisture levels and population densities used in the experiment are such that the microorganisms are required to traverse a short distance from their habitat in the soil to the root, then an actively motile organism would gain a benefit over one that must rely on passive diffusion. Thus, it can be concluded that directed motility (i.e. chemotactic attraction) is most important to nodulation once the microbe has been moved passively by mass water flow into the vicinity of the susceptible portion of the root. It is assumed that flowing water is more important in moving the bacteria over distances up to several centimeters or more whereas microbial motility primarily impacts interactions of the bacteria with roots for cells located within a few millimeters or less from the root.

This model of the relative importance of passive and active motility of bacterial strains in nodule formation rests upon the assumption that rhizobia are capable of responding chemotactically to root exudates. Rhizobia are attracted to root exudate components (e.g. Bhagwat and Thomas 1982). Exudate components found to attract various rhizobial strains include sugars and amino acids (e.g. Bergman et al. 1988), proteins (e.g. Currier and Strobel 1977), dicarboxylic acids (e.g. Barbour et al. 1991), aromatic compounds (e.g. Kape et al. 1991), and flavonoids (e.g. Dharmatilake et al. 1992). (It should be noted that attraction to the general organic chemical components of root exudates is not specific to the rhizobia, but a variety of rhizosphere bacteria are attracted to the root by these compounds. To a major degree, the response of the rhizobial population to root exudates is part of a more generalized stimulation of bacterial population densities in the rhizosphere.)

There is an impact of some root exudate components and expression of nodulating genes by the *Rhizobium* cell. Deletions in the *nif-nod* region of the symbiotic megaplasmid eliminated the chemotactic attraction of *R. meliloti* to plant roots by the flavonoid luteolin (Caetano-Anollés et al. 1988). The *nif-nod* region is constituted of

genes involved in nodule formation and nitrogen fixation. For further discussion of the induction of these genes by components of root exudates and their role in nodule initiation, see Brewin (1991).

Host recognition and attachment: Reaction to root exudate components may allow the rhizobial strains to reach the specific portion of the root structure most susceptible to *Rhizobium* infection (that portion of the root behind the apical meristem at the site of emergence of the root hair, i.e. the zone of root elongation) more efficiently. The next step in nodule formation requires recognition and attachment to the root. Recognition of susceptible host tissue involves an interaction between proteins (lectins) on the plant root surface and rhizobial exopolysaccharides. This process could be likened to the specificity and interactions associated with an antibody–antigen reaction. Early suggestions of the role of lectins in the recognition process were presented by Bohlool and Schmidt (1974). They found that a lectin from soybean combined specifically with the soybean nodulating bacterium (*B. japonicum*) and that this lectin did not combine with other representative rhizobia. Sherwood et al. (1984) found that the location of the lectin-binding polysaccharides of *R. trifolii* on the cell surface varied with age of the culture. Random distribution of the binding agent as well as polar concentration of the substance were detected after different periods of cellular growth. The quantity and location of these receptors on the bacteria directly correlated with their attachment in short-term studies of clover root hair binding. For example, cells from three- or 21-day-old cultures attached nearly exclusively in a polar fashion whereas five-day-old cells with the binding factors randomly distributed on the cell surface attached randomly to the root tissue.

Rhizobial exopolysaccharides appear to serve multiple purposes in nodulation (see Reuber et al. 1991 for a more detailed discussion of the role of exopolysaccharides in nodule formation). The varied roles include assistance in entangling the bacterial cell in the mucigel of the plant root, inhibition of host defenses through masking of the bacterial surface, and encapsulation of the bacterial cell for its protection in the infection thread. For example, after attachment and orientation of the *R. trifolii* cell with the clover root surface, an accumulation of extracellular microfibrils associated with lateral and polar surfaces of the bacterial cell occurs (Dazzo et al. 1984). The formation of these fibrils results in an attachment of the bacteria to the root hair sufficiently strong to withstand the hydrodynamic shear forces associated with high-speed vortexing of the mixture. (See Brewin 1991 for further discussion of the role of these substances in nodule formation.)

Nodule formation: Once the rhizobial cell has attached to the legume root, an infection thread spreading down the root hair is formed. Development of this structure is preceded by deformation of the root hair that is induced by exopolysaccharides formed by the rhizobial cell (e.g. Battisti et al. 1992; Ervin and Hubbell 1985; Higashi and Abe 1980; van Brussel et al. 1992). The invagination of root hair

wall forming the cellulosic infection thread allows the bacterial cells to invade into the central portions of what becomes the nodule. The invading rhizobial cells pass through the infection thread to the root cells adjacent to the root hair. These root cells subsequently become infected by the invading rhizobial cells. Only tetraploid cells are infected and become the primordia of the nodule. It must be noted that only a small portion (usually less than 5%) of the invaded root hairs develop nodules. Division of the infected cells results in formation of the nodule. Bacteria multiply within the tetraploid cells, forming swollen, variable shaped and branched cells known as *bacteroids*, that are separated into packets within the nodule by a *peribacteroid membrane*. There are typically 4–6 bacteria per packet. Nitrogen fixation within the root nodules only occurs after formation of the bacteroids.

The nodulation cycle could be said to be completed by death and deterioration of the nodule and release of the bacteroids and bacteria contained therein into the soil. The bacteroids are apparently incapable of division when released into soil by death and decay of the nodule. Data collected by Paau et al. (1980) indicate that *R. meliloti* bacteroids in alfalfa nodules are degraded during nodule senescence. The nodule apparently always contains small numbers of dormant rod-shaped cells that are capable of existence free in the soil environment.

13.1.4 Plant Control of Nodule Formation

The host plant is not a passive participant in this process involving "rearrangement of its architecture" by the *Rhizobium* strain. Expression of several plant genes is necessary for establishment of the symbiotic interaction. In their survey of the literature, Caetano-Anollés and Gresshoff (1991) provide a listing of symbiotic legume mutants for soybean (*Glycine max* [L.] Merr.), alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), crimson clover (*Trifolium incarnatum* L.), pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), and peanut (*Archis hypogaea* L.). For some of the legumes, only one or two genes impacting nodulation have thus far been elucidated, but a total of 22 genes interacting with the bacterial symbiont in nodule formation were listed for pea. These observations support the conclusion that the genetic-based contribution of the host plant to root nodule formation is as or more complex as that of the invading bacterium.

An interesting point of control by the host plant is its determination and optimization of the number of nodules formed. Most obvious among these properties is the impact of untimely fertilization of the crop on nodulation. Such inappropriate addition of fixed nitrogen to the crop suppresses nodule formation.

A less appreciated interaction is autoregulation. With autoregulation of nodulation, the plant controls the quantity and timing of nodule formation. After the first infection of roots, no further increase in number of root nodules occurs. The benefit to the host plant in preventing formation of unnecessary nodules is obvious

since nodulation requires the commitment of large quantities of plant resources. This inhibition of nodulation is reversible in that removal of nodules can induce a new round of nodule formation. This process requires expression of specific, as yet undescribed plant genes.

Furthermore, the rhizobia involved in the first round of infection must have the genetic capability to induce nodule formation and to grow within the nodules. George and Robert (1991) assessed the capacity of a number of mutants of *R. leguminosarum* bv. phaseoli to induce an autoregulatory response of *Phaseolus vulgaris* L. They found that all mutants unable to form nodules failed to induce a suppressive response of the host. The host response correlated with the ability of the bacteria to grow inside the nodules but not with the capacity to initiate nodule formation or to fix nitrogen. That is, the host response required that the infecting bacterium be infective, but not effective.

The interaction of autoregulatory responses and bacterial population density to yield optimal nodulation has been examined in soybean (*G. max* [L.] Merr. cv. Bragg) following inoculation with *B. japonicum* USDA 110 (Caetano-Anollés and Gresshoff 1991). The roots were initially susceptible to infection 3–4 days following germination. The number of nodules formed was proportional to the bacterial density up to an optimal concentration. The maximal population density resulting in an increase in the quantities of nodules formed was greater for a supernodulating soybean variant than was observed with the wild type. Thus, the plant genome can be manipulated to increase nodulation of this agronomically important crop.

13.2 Manipulation of *Rhizobium*–Legume Symbioses for Ecosystem Management

The foregoing evaluation of the *Rhizobium*–legume symbiosis suggests the existence of a relationship that is of interest not only for basic studies but also for a variety of practical applications. The ubiquity of legumes and their significance as food crops suggest both economic and environmental benefits from management of this interaction. Prime questions in such considerations are as follows.

- Do *Rhizobium*–legume symbioses add significant quantities of fixed nitrogen to a soil ecosystem?
- Is there potential to improve the nitrogen inputs in a manner that will also augment ecosystem productivity?
- Are these improvements feasible with currently available methods?

Encouragement of *Rhizobium*–legume symbioses development does increase soil fixed nitrogen resources. The balance between nitrogen inputs and outputs to

land systems and the identity of the sources of this fixed nitrogen (Burns and Hardy 1975) support the conclusion that *Rhizobium*–legume symbioses are a major source of fixed nitrogen in land-based systems. About 64% of the 139×10^9 kg N_2 biologically fixed in terrestrial systems is produced in agricultural systems. Since the bulk of biological nitrogen fixation in agricultural systems is derived from the cultivation of legumes, these symbioses can be concluded to provide well over half of the biological source of fixed nitrogen. Data collated by Alexander (1977) provide a prospective on fixed nitrogen contributions of individual crops on a more localized basis. Inputs of fixed nitrogen for alfalfa, red clover, pea, soybean, cowpea, and vetch range from 65 to 335 kg N_2 fixed ha^{-1} yr^{-1}.

Once it has been concluded that these symbiotic associations are major contributors of fixed nitrogen in soil systems, the point of concern must be redirected to a consideration of the potential to increase fixed nitrogen inputs by optimization of this process. That is, the possibility of improving plant biomass yields or plant community sustainability through use of more efficient combinations of rhizobia and legumes must be examined. A diverse population of rhizobial strains already exists throughout the soil profile in agricultural and nonagricultural soils: a leguminous tree, black locust (*Robinia pseudoacacia* L.) (Batzli et al. 1992); *R. leguminosarum* in agricultural soil (e.g. Bottomley and Dughri 1989; Brockman and Bezdicek 1989); soils under acacia trees (*Acacia albida*) (Dupuy and Dreyfus 1992); soils from an alfalfa field (Jenkins et al. 1987); the woody legume, mesquite (*Prosopis glandulosa*) in desert soils (Jenkins et al. 1989; Waldon et al. 1989), and in tropical soils of Hawaii (Woomer et al. 1988). Furthermore, the indigenous rhizobial population can be quite diverse. For example, Noel and Brill (1980) used sodium dodecyl sulfate (SDS) gel electrophoresis to evaluate nodule occupants from soybeans collected from two Wisconsin (USA) locations. At one location, 19 gel types were distinguished and classified into three groups.

Nodule occupancy by these indigenous rhizobia is not the result of superior nitrogen-fixing capacity. It is derived more from a capacity of the bacterial strain to survive in soil. Additionally, both the population density and location of the bacterial habitat must allow optimal contact with susceptible roots. The proportion of the total soil rhizobial population represented by any individual strain is controlled by soil properties as well as plant growth traits. For example, the predominance of any particular rhizobial strain in the soils studied by Noel and Brill (1980) depended on the time and depth of nodule formation on the host plant. Also, Mahler and Bezdicek (1980) found that serogroup variability of *R. leguminosarum* strains in soils was affected by soil temperature, moisture, and position on the slope in the catena. Both microclimate and macroclimate variation altered strain distribution of *R. leguminosarum* strains in peas (*P. sativum* L.) in a Palouse (Washington, USA) site. Subsequent studies of 192 *R. leguminosarum* strains from this site showed that they grouped into three serogroups and 18 plasmid profile

groups. Similar variety and distribution have been observed for *R. meliloti* (Jenkins and Bottomley 1985). The latter individuals identified a variety of protein profiles for *Rhizobium* isolates from alfalfa (*M. sativa* L.) with SDS-gel electrophoresis.

Thus, it can be concluded that in any given soil system supporting growth of legumes, a variety of rhizobial strains are available for nodulation of root tissue. The adequacy of this indigenous population to form optimal numbers of nodules on legume populations generally depends on plant density. This principle is best exemplified by contrasting native and intensively cultivated legumes. In native ecosystems, indigenous rhizobial population densities are generally sufficient to support adequate nodulation of leguminous species to maintain ecosystem fertility and biomass productivity. With high yield, cultivated agricultural systems or with monoculture systems, such as alfalfa fields, nitrogen fixation is commonly seen to be augmented by inoculation with exogenously grown rhizobia. Therefore, it is reasonably concluded that indigenous soil population densities were insufficient to support optimal nodulation. Thus, addition of laboratory-produced or axenically grown field *Rhizobium* sp. isolates to soils and legumes seeds to stimulate crop productivity has long been a common agricultural practice. The increased nodulation of the legume roots and subsequent augmentation of biologically produced fixed nitrogen result in part from the higher probability of encounter between the host root and infective bacteria as well as the utilization of rhizobial isolates selected for enhanced nitrogen-fixing capability.

13.3 Rhizobial Inoculation Procedures

Primary concerns in analysis of the benefits of manipulation of soil rhizobial populations have been (i) development of an effective delivery system, (ii) determination of the interactions with the soil environment and soil microbial population affecting survival of the inoculant, and, more recently, (iii) elucidation of properties of the inoculant strain affecting its competition with indigenous rhizobial variants for nodule occupancy. (See Brockwell and Bottomley 1995 for a review of this topic.)

13.3.1 Inocula Delivery Systems

Axenically grown rhizobial strains capable of efficient nitrogen fixation are of little value if they do not form effective associations in the field. The first step in effective use of laboratory-derived/produced rhizobial strains is the physical conveyance of viable bacterial cells to the soil. In a scientific experiment, this step is reasonably easy since a microbiologist can quite easily prepare an active bacterial

culture that can immediately be added to crop roots or seeds. Difficulties are encountered in transferring this "technology" into a "real-world" situation where the viable bacterial cells may be abused tremendously before introduction to their new environment. A commercially prepared inoculum must be capable of surviving for long periods of time under somewhat adverse conditions between growth and delivery to the agriculturist and of enduring potential maltreatment on site before the legume seeds are inoculated.

A variety of delivery systems for rhizobial inocula have been evaluated and implemented in field situations. Examples include ground peat or an oil carrier (Kremer and Peterson 1983) with or without fertilizer (Kremer et al. 1982), irrigation water (Ciafardini et al. 1992), water (Crist et al. 1984), coal-based carriers (Crawford and Berryhill 1983), soils (Chao and Alexander 1984), and fluid gels (Jawson et al. 1989). A variety of commercial inoculants are available (e.g. Hume and Blair 1992). Variables that must be evaluated include pretreatment of the support media (e.g. sterilization of the peat [Strijdon and van Rensburg 1981]), and methods to prepare the inoculum for storage (e.g. drying of inocula and humidity for storage [Mary et al. 1985]).

13.3.2 Survival of Rhizobial Inocula

A related aspect to optimization of fixed nitrogen inputs from *Rhizobium*–legume symbioses is maximization of inoculum survival once they are amended to the soil system. Although the bacterial strain or variant selected for legume inoculation may have been derived from the soil–plant environment, cultivation under laboratory conditions necessarily induces changes in the phenotypic and genotypic properties of the cell. These alterations may or may not affect the capacity of the organism to adapt to the soil environment and to compete with indigenous populations. Axenically grown laboratory rhizobial strains added to legume seeds or amended directly to soil are stressed by the physical and chemical properties of the harsh soil environment as well as by interactions with the general soil microbial community. The capacity of the organism to overcome these barriers to survival determines its probability of existing in the soil site for sufficient duration to encounter a susceptible legume root and initiate infection.

Rhizobial strains amended to soils may survive for several years. For example, Crozat et al. (1982) found that their strains of *R. (Bradyrhizobium) japonicum* introduced into a variety of French soils survived at high rates ($\geq 10^4$ bacteria g^{-1} dry soil after five years). Similarly, Brunel et al. (1988) found that *B. japonicum* USDA strains 125-Sp, USDA 138, and USDA 138-Sm survived for 8–13 years following their release into soil. Alien rhizobial strains (i.e. axenically grown and introduced or reintroduced into the soil system) persisted by passage through legume nodules produced in the soil. Survival of a portion of the introduced population may result from

a capacity to adapt to the prevailing soil conditions and to compete with indigenous microbes. Viteri and Schmidt (1987), using fluorescent antibody techniques to study variation in population densities of *B. japonicum* seroclusters 123 and serogroups 110 and 128, found that amendment of soil samples with metabolizable sugars resulted in not only an increase in the general microbial populations but growth of the various rhizobial strains. This observation supports the conclusion that rhizobia are capable of competing with the general soil flora. That is, these bacteria do not require the exclusive protection of the plant nodule to maintain soil populations.

13.3.2.1 Controllers of Diazotroph Growth

Primary soil physical and chemical properties affecting survival and nodule formation which are of concern to environmental microbiologists include soil pH, metal interactions, salinity, and moisture. Although the effects of these soil properties on nodulation have been most studied with agronomically important crops, they are also a major consideration when developing procedures to encourage fixed nitrogen inputs into reclaimed or managed ecosystems. It is generally desirable to minimize anthropogenic intervention in reclamation sites. That is, it is useful to develop site-resident biological mechanisms for control of fixed nitrogen inputs rather than to rely on long-term fertilization plans. Thus, selection and control of an indigenous biological population capable of fixing nitrogen is essential.

13.3.2.2 Rhizobia and Acid Soils

Soil acidity affects the legume plant, the infecting rhizobia, and the product of their interaction – the symbiotic association. Generally, host plant growth is less sensitive to extremes of acidity than is the *Rhizobium* species. Infection of host roots and survival of the bacterial symbiont are generally limited to pH greater than 5.0 whereas the host plants may grow at values as low as 4.0. This pH sensitivity is of concern to the agricultural community because of the need for sustained growth of symbiotic associations in acid soils, as well as to the environmental science community in order to determine the potential for use of these highly effective fixed nitrogen sources in acid-impacted sites, such as soils receiving acid mine slags or drainage. Examples of agricultural concerns include the difficulties associated with maintenance of alfalfa (Barber 1980; Lowendorf and Alexander 1983a), lucerne (Munns and Fox 1977; Pijnenborg et al. 1991), and clover (Richardson et al. 1988a) stands in acidic soils. Similar sensitivity to acidic soils has been observed for tropical legumes (18 legume species) as occurs with temperate legumes.

Both direct and indirect effects of acidic conditions on rhizobial activity are observed. Bacterial cells are affected by the elevated hydrogen ion concentration of acidic soils (direct effect) as well as the alteration of metal solubility resulting from reduction in the soil pH (indirect effect) (e.g. Franco and Munns 1982; Keyser and Munns 1979a). The latter interaction can be due to increased metal

concentrations (e.g. inhibition of growth by augmented aluminum concentrations under acidic conditions) or result from decreased soluble metal levels (e.g. reduction of the essential mineral molybdenum by increased acidity).

A differential sensitivity of the rhizobial strains to the various changes in soil cation concentration resulting from increased soil acidity has been observed. Keyser and Munns (1979b) in a study of cation effect on growth of *R. (Bradyrhizobium) japonicum* found that aluminum toxicity and acidity itself had a greater impact on rhizobial growth than did manganese toxicity and calcium deficiency. It must be noted that aluminum toxicity and acid sensitivity independently affect rhizobial survival in soil (e.g. Hartel and Alexander 1983; Hartel et al. 1983). It appears that survival of cowpea (*Vigna unguiculata* [L.] Walp.) rhizobia is pH but not aluminum sensitive. Hartel et al. (1983) concluded that aluminum content of soil was not a major factor controlling cowpea rhizobial survival but it did significantly alter the nodulation process.

Although it has been proposed that survival of indigenous populations of *R. trifolii* in extremely acidic soils can occur through refuge in soil microsites of less extreme acidity (Richardson and Simpson 1988), rhizobial strains with increased tolerance of acidic conditions can be isolated (e.g. Indrasumunar et al. 2011; Keyser et al. 1979; Lowendorf and Alexander 1983b; Thornton and Davey 1983; Tiwari et al. 1992) and constructed genetically (Chen et al. 1991). Increasing acid tolerance of rhizobial strains under axenic condition does result in augmented survival in sterile acid soils (Lowendorf et al. 1981). Thus, the potential exists to select or engineer rhizobial strains in the laboratory that are both efficient nitrogen fixers and acid tolerant for use in acid-impacted soil sites. Augmented survival in the acid soils could increase the probability of nodule occupancy (Dughri and Bottomley 1983). Furthermore, interactions between the acidic conditions and cation concentrations can also affect nodule formation by rhizobia. For example, acidic conditions and levels of calcium and aluminum ions reduce induction of the nodulation genes in *R. leguminosarum* bv. *trifolii* (Richardson et al. 1988b). Thus, it can be concluded that the concentrations of fixed nitrogen provided by the *Rhizobium*–legume symbioses in acidic and/or cation-impacted soils depend at least in part upon the capacity to maintain adequate population densities of infective bacteria in the soil for optimal encounter between the bacterium and susceptible plant roots.

No single cell property can be selected to optimize the acid tolerance or aluminum sensitivity of rhizobia. For example, DNA repair mechanisms (Johnson and Wood 1990), extracellular polysaccharide production (Cunningham and Munns 1984a), and ability to maintain intracellular pH (O'Hara et al. 1989) all appear to affect aluminum and/or acid tolerance of rhizobial strains. Variation between rhizobial strains is exemplified by studies of the role of extracellular polysaccharide production in protection of the bacterial cell. Cunningham and Munns (1984a) found that acid tolerance of rhizobial strains for six legume genera (*Cicer*,

Phaseolus, *Leucaena*, *Lens*, *Melilotus*, and *Trifolium*) all correlated with extracellular polysaccharide production. The quantities of extracellular polysaccharide produced by 20 strains of *R. phaseoli* varied from 18 to 75 mg per 100 mL culture. The amounts of polysaccharide produced correlated with acid tolerance. Data from further studies (Cunningham and Munns 1984b) indicated that the apparent protection did not relate to buffering capacity of the extracellular polysaccharide or its ability to chelate aluminum ions. In contrast, Kingsley and Bohlool (1992) evaluated the role of extracellular polysaccharide produced by *R. leguminosarum* bv. *phaseoli* (CIAT899) with mutant strains incapable of producing the polysaccharide. No difference in aluminum tolerance of the wild-type strain from the mutant was detected. Results from this study indicate that neither intracellular or extracellular products of the *R. leguminosarum* strain alleviated the aluminum toxicity of sensitive rhizobial strains. These studies demonstrate the potential to increase the utility of the *Rhizobium*–legume symbioses for augmenting fixed nitrogen inputs into acidic soil ecosystems as well as the complexity of the genetic control of this acid and aluminum sensitivity of the infective bacteria.

Although most interest in the effect of toxic metals on nitrogen fixation by *Rhizobium*–legume associations has involved growth of agriculturally important legumes in acidic soils, some consideration of the impact of metals is necessary in evaluating the use of these associations in metal-impacted sites or in soils amended with materials that may contain environmentally significant levels of metals (e.g. Dahlin et al. 1997; McGrath et al. 1988; Smith 1997). For example, McGrath et al. (1988) determined the potential for reduction of fixed nitrogen yields from nodulated *Trifolium repens* in soils receiving metal-contaminated sewage sludge. Plants grown on the contaminated sludge-amended soil had reduced nitrogen concentrations and reduced yields compared to those produced on uncontaminated control soils. The root nodules on the impacted plants were numerous but ineffective in nitrogen fixation.

13.3.2.3 Soil Moisture Impact on Diazotroph Populations

The occurrence of rhizobial populations in desert soils and the effective nodulation of legumes growing therein (e.g. Jenkins et al. 1987, 1989; Waldon et al. 1989) attest to the fact that rhizobia are capable of existing in soils with low moisture levels. Population densities do tend to be lowest under the most desiccated situations, with increasing densities as the moisture stress is relieved. The wide range of moisture levels characteristic of ecosystems where legumes have been shown to fix nitrogen suggests that rhizobial strains with varying sensitivity to soil moisture can be selected. Laboratory studies have shown that sensitivity to moisture stress varies with bacterial strain with a variety of rhizobial strains and species (e.g. with *R. leguminosarum* bv. *trifolii* [Boonkerd and Weaver 1982; Fuhrmann et al. 1986], with *R. meliloti* [Busse and Bottomley 1989], with cowpea rhizobia [Osa-Afiana

and Alexander 1982a], and with *B. japonicum* [Mahler and Wollum II 1980]). Thus, it is reasonable to assume that rhizobial strains can be selected with moisture stress tolerance within the range of growth of its legume host.

The moisture tolerance of rhizobia is also affected by other soil properties or components. For example, survival of rhizobia in drying soils can be extended by increasing contents of soil organic matter and decreased by elevated soluble aluminum (Chao and Alexander 1982) and by the proportion of clay in the soil (Osa-Afiana and Alexander 1982b). Furthermore, organisms produced *in situ* tend to be better adapted to moisture stress than axenically grown organisms (Pena-Cabriales and Alexander 1979). Optimization of soil moisture for growth of the host plant, which generally is more sensitive to moisture stress than is the bacteria, results in maximization of development of *Rhizobium*–legume fixed nitrogen inputs into the soil system.

13.3.2.4 Soil Salinity

An allied concern in regard to soil moisture in reclamation of environmentally impacted sites can be the effect of salinity on survival of rhizobia in soil systems. For example, amendment of soils with products of our industrial society can raise the soil salinity (e.g. sludge-borne salts [Madariaga and Angle 1992]). As indicated in Chapter 5, increasing salts may have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stresses.

Rhizobia vary in efficacy of osmoregulation – response to salt stress (e.g. Busse and Bottomley 1989; Surange et al. 1997; Zahran 1997). This adaptation of the microbial population was exhibited in a study of the survival of *B. japonicum* in sludge-amended soils. The soluble salts of the sludge (not the heavy metals) were shown to be primarily responsible for a short-term reduction in bradyrhizobial populations following sludge application to soil. Singleton et al. (1982) found many *Rhizobium* strains with enhanced salinity tolerance compared to most agriculturally important varieties. In a study of distribution of fast-growing and slow-growing mesquite nodulating rhizobia in desert ecosystems, it was found that distribution of the rhizobial strains within the rooting system was related to total soil salts (Jenkins et al. 1989).

This analysis of some soil properties affecting survival, growth, and nodulation capacities of soil rhizobial populations demonstrates the versatility and adaptability of these essential soil populations. The genotypic and phenotypic flexibility exists within these organisms to adapt to many of the stressed soil conditions normally encountered in our soil system. This observation does not imply efficient nitrogen-fixing capabilities under these conditions. The legume may be nodulated, but fixed nitrogen production may be below the level required for optimal growth of the host plant. In these situations, symbiotic associations, such as the actinorhizal interaction, may be better adapted or more adaptable for use in site management or reclamation.

13.3.3 Biological Interactions in Legume Nodulation

Rhizobia are capable of growth in native soil samples when the soils have been amended with fixed carbon sources supportive of their metabolism (e.g. Pena-Cabriales and Alexander 1983a; Viteri and Schmidt 1987) or when they receive nutrients from growing roots (e.g. Pana-Cabriales and Alexander 1983b). Success of this nutrient-based competition with indigenous populations depends on the capacity of the alien microbe to become established in an unoccupied niche.

Rhizobia are best suited for competition for the newly created niche of the nitrogen-fixing nodule. Life as a member of the "free-living soil community" is more difficult. Rhizobia not only must compete for limited nutrients, but interactions with indigenous heterotrophic microbes and predators reduce their capacity to maintain population densities at sufficient levels to assure contact with susceptible legume roots (e.g. Li and Alexander 1986; Pugashetti et al. 1982; Ramirez and Alexander 1980). The outcome of competition between indigenous microbial can be manipulated. Aside from the brute force method of overwhelming the system with massive population densities of the laboratory-grown rhizobial strain, use of fungicides and antibiotics can enhance the probability of rhizobial colonization (Hossain and Alexander 1984; Li and Alexander 1986). Hossain and Alexander (1984) augmented soybean rhizosphere colonization with *B. japonicum* through use of benomyl (a fungicide) and the antibiotics streptomycin and erythromycin. *Rhizobium* strains used with these procedures must be resistant to the inhibitors. Unfortunately, amendment of the soils with specific antibiotics may also result in the development of antibiobic-resistant microbial strains.

13.4 Nodule Occupants: Indigenous vs Foreign

Two sources of rhizobia for root infection can occur in many managed agricultural soils: (i) native populations and (ii) crop inoculants. The laboratory-produced rhizobial strains used to inoculate crops must be capable of competing for nutrients with indigenous populations until they encounter a susceptible root. Additionally, they also participate in an intense race with native strains for occupancy of nodules. A study of the history of use of *Rhizobium* inoculants for legume cropping suggests that it has been assumed that the soil-amended strain necessarily predominated in nodule occupation. Perhaps a more pragmatic assessment of the situation would include the idea that the investigator and growers have not been as concerned with the identity of the nodule occupant as they were with augmentation of fixed nitrogen resources and the concurrent improvement in crop yields. If crop yields increased, a benefit from inoculation with highly efficient rhizobial strains was assumed to have occurred. Unfortunately, detailed examination

of legume nodules reveals that it is not unusual for the laboratory-cultivated rhizobial strains to lose the competition for a place in the nodule to the less efficient but better adapted indigenous soil populations.

Nodule occupancy frequencies for nonnative rhizobial strains ranging from nearly zero to essentially 100% have been reported. For example, van Rensburg and Strijdom (1985) compared *Rhizobium* nodule occupancy frequency of indigenous and axenically grown strains in *Trifolium* spp., *Medicago* spp., *G. max*, and *Lotus pedunculatus*. They collected 420 nodules from crops growing in fields that had been inoculated 4–8 years previously with highly efficient rhizobial strains. The rate of nodule occupancy for the inoculated strain ranged from 17.7% in *G. max* (soybean) to 100% with *L. pedunculatus*. Klubek et al. (1988), in a study of *B. japonicum* nodule occupancy in the Midwestern USA, detected frequency of occurrence of inoculated strains as low as 0.3%. Detection of highly variable capacity of laboratory-grown strains to compete with indigenous populations is commonly reported (e.g. Materon and Hagedorn 1992; McDermott and Graham 1989; Moawad and Bohlool 1984).

The most obvious explanation for the variation in frequency of nodule occupancy of soil-amended rhizobial strains involves the probability of encounter of the added organism with susceptible portions of the legume root. In a study of seven legume species in Hawaii (Thies et al. 1991a), it was shown that the proportion of the nodules occupied by inoculated rhizobial strains depended on the population density of the indigenous population. Indigenous organisms would be anticipated to be better adapted to the soil system and perhaps even more appropriately distributed within the soil profile than alien organisms. The latter conclusion is based upon the observation that the physical location of indigenous organisms depends in part on the distribution of the root system in which they grew and the homogenization of the soil associated with intensive cultivation systems. Thies et al. (1991b) found that 59% of the observed variation in inoculation response could be attributed to the population density of the indigenous population.

Properties of rhizobia that enhance nodule occupancy rates are diverse and, to a major extent, poorly defined. A primary requirement is the ability to maintain sufficient population densities in the soil to allow a reasonable probability of encounter with host roots. Therefore selection of inoculant strains must include consideration of the properties of the soil in which they are to be used (e.g. moisture/salinity stresses, pH) as well as the ability to compete with indigenous microbes for available nutrients and to withstand incursions of predators and parasites. Further considerations relate the efficiency of the nodulation process. Initiation and development of effective nodules is a complex process. Many of the rhizobial cells attracted to the appropriate portion of the legume root fail to complete the infection process. To increase the probability of the inoculant with improved nitrogen-fixing efficiency contributing to augmentation of ecosystem

fixed nitrogen resources, variants with increased capability of completing the nodule formation process are desirable.

13.5 Actinorhizal Associations

When the potential to form nitrogen-fixing nodules on plant roots is considered, the *Rhizobium*–legume association is frequently discussed, nearly at the exclusion of considering any other interactions, yet actinorrhizal interactions (nonleguminous nitrogen-fixing symbiotic associations between actinomycetes of the genus *Frankia* and a variety of woody angiosperms) are major contributors to nitrogen inputs in forests, wetlands, fields, and disturbed sites of temperate and tropical regions. Actinorhizal associations involve more than 160 species of angiosperms classified among six or seven orders. As shown in Table 13.1, a wide variety of genera of angiosperms are capable of forming actinorhizal associations. Not all genera in the various families cited are active in nitrogen fixation and not all species of the cited genera form nitrogen-fixing associations.

Contributions of fixed nitrogen to native as well as managed ecosystems by these symbioses are comparable to those of the more studied *Rhizobium*–legume interactions. The nitrogen gains due to these interactions vary with soil, climate, and plant age. Typical contributions represented by *Alnus* associations are

Table 13.1 Examples of angiosperm families involved in nitrogen-fixing actinorhizal associations

Family	Genera
Betulaceae	*Alnus*
Casiaromaceae	*Casuarina*
Coriariaceae	*Coriaria*
Elaeagnaceae	*Elaeagnus* *Hypophae* *Shepherdia*
Myricaceae	*Myrica* *Comptonia*
Rhamnaceae	*Ceanothus* *Discaria*
Rosaceae	*Cercocarpus* *Dryas* *Pushia*

12–2000 kg N ha^{-1} and *Hippophae* (27–179 kg N ha^{-1}). See Baker and Mullin (1992) for a review of the contributions of this association to global nitrogen cycling.

Furthermore, the energy requirements (i.e. photosynthetate contributions from host plants) and nitrogenase activity of actinorhizal symbioses are comparable to those of the *Rhizobium*–legume associations (Gauthier et al. 1981; Tjepkema and Winship 1980). Note that as with rhizobia, *Frankia* strains are capable of fixing nitrogen growing in axenic culture on defined media (Gauthier et al. 1981).

It has been recognized that the microbial symbiont is an actinomycete for more than 100 years. Early identification was based on microscopic detection of actinomycete-like mycelial structures in nodule tissue (see Quispel 1990 for a discussion of this topic). The greatest limitation to study of actinorrhizal associations has been, until recently, the inability to culture the microbial partner exclusive of the plant nodule. Until the early 1980s, the microbial partner was generally regarded to be an unculturable actinomycete. Most hypotheses regarding participation of the microbial symbiont were therefore developed from observation of the cells in the nodule or from biochemical studies of partially purified microbial biomass extracted from crushed nodules.

The *Frankia* sp. can now be isolated and cultured (e.g. Baker et al. 1979; Benson 1982). Procedures generally involve surface sterilization of the nodules with such substances as chlorox, hydrogen peroxide, or dilute silver nitrate. The sterilant is washed from the nodules and the nodules are crushed. The extraneous debris may be removed (Baker et al. (1979) used sucrose gradients) or laboratory culture media may be inoculated directly with the nodule extract. A variety of nitrogen-free media containing antibiotics have been used to culture the actinomycete. A key in culture of these organisms is the necessity of inhibiting the growth of fast-growing contaminants. Colonies of the slow-growing *Frankia* spp. are easily overgrown by faster growing microbes also contained in the inocula. Thus, antibiotics such as cycloheximide and nystatin are amended to the growth media (e.g. Benson 1982). Once isolated, the proof that the axenically grown actinomycete participates in symbiotic produced fixed nitrogen results from successful nodulation of uninfected host plants with the cultured microbial strain (e.g. Diem et al. 1982; Mansour et al. 1990; Rogers and Wollum 1974; van Dijk and Sliumer-Stolk 1990).

Polymerase chain reaction amplification and related DNA analytical techniques may be valuable tools for elucidation of the diversity of *Frankia* strains involved in actinorhizal symbioses (e.g. Beayzova and Lechevalier 1992; Sellstedt et al. 1992; Simonet et al. 1990, 1991) as well as the variability of these nitrogen-fixing actinomycetes in soil (e.g. Hahn et al. 1990a; Hilger and Myrold 1991; Myrold et al. 1990) and in host nodules (Bloom et al. 1989; Hahn et al. 1990b).

Although at this time it is not recommended that the genus *Frankia* be divided into individual species (Lechevalier and Lechevalier 1989), axenically grown

Frankia strains are extremely diverse. For example, DNA hybridization studies (Bloom et al. 1989) revealed highly divergent populations of *Frankia* could be isolated from bayberry (*Myrica pensylvanica*) nodules. In fact, *Frankia* strains isolated from the same nodule were shown to be not only phenotypically different but also genotypically diverse. Similarly, restriction fragment length polymorphism analysis has been used to divide *Frankia* strains into groups that correlate with host specificity groups separated by cross-inoculation studies (Nittayarjarn et al. 1990) (As with rhizobia, *Frankia* strains can be separated into groups based on the specificity toward their host plant.)

13.6 Conclusions

Root nodule-forming symbiotic associations are primary producers of fixed nitrogen in terrestrial ecosystems. Both rhizobial and *Frankia* sp. strains form specific associations with legume and angiosperm hosts, respectively. Although adequate populations of infective diazotrophs commonly occur among soil microbes, the potential exists for manipulation of these bacterial populations to increase their contribution of fixed nitrogen to the ecosystem. For rhizobial populations, it has been shown that sufficient genotypic variability exists for resistance to soil pH, moisture, and salinity extremes as well as for nitrogen-fixing efficiency to allow for development of rhizobial strains that will support increased fixed nitrogen inputs compared to those provided by the activity of indigenous populations. Selection of such strains and their utilization in the field also require consideration of those phenotypically expressed traits that improve the microbes' capacity to compete with other soil herotrophs as well as indigenous rhizobia.

With the application of newly developed DNA-based procedures, understanding of the genotypic varability of soil diazotrophs and their contributions to fixed nitrogen pools in both native and anthropogenically managed soils is greatly enhanced. Examples of such studies include (i) a large diversity of non-rhizobial endophytes as well as nonclassical rhizobia have been detected in soil (de Meyer et al. 2011, 2015; Howieson et al. 2013); (ii) novel strains of diazotrophs have a role in nitrogen fixation in acidic and nutrient-poor soils (Garau et al. 2009; Magadlela et al. 2017); and (iii) geographic location has a significant effect on genetic diversity and composition of *R. leguminosarum* (van Cauwenberghe et al. 2015). As our understanding of the ecological associations of *Frankia* spp. develops, similar manipulations to increase fixed nitrogen production by actinorhizal symbiosis in managed and wild-land ecosystem should be possible.

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14

Denitrification

Some would use a term such as *pollutant* when considering the occurrence of nitrate in soil whereas others may prefer to describe soil nitrate as an essential nutrient for ecosystem sustenance. One group would seek to minimize levels of the alleged environmentally offensive substance, whereas those interested in plant biomass production would seek to optimize nitrate production. Both groups would underscore the necessity of understanding processes leading to losses of soil nitrate to achieve their objectives.

To a great degree, the existence of such divergent opinions regarding the value of nitrate in soil results from the propensity of this compound to be in the wrong place at the wrong time at undesirable concentrations. Nitrate ions in the root zone of a food crop during maximal biomass production are inarguably beneficial whereas migration of that same nitrate below the root zone to underlying aquifers transforms a beneficial soil chemical component into an undesirable water contaminant.

Societal implications associated with the timing and location of accumulation of significant soil nitrate loadings reach far beyond this conflict of development of a soil nitrate balance sheet and agreement on the best nitrate management plans for maintenance of an ecosystem. For example, traditional considerations have included concerns that nitrate contents of soil leachates or runoff waters may alter regional water quality (e.g. augmented lake eutrophication rates) and that there are potential public health difficulties (methemoglobenemia) associated with elevated nitrate loadings in drinking waters. Other concerns include management of fixed nitrogen amendment to soil systems to minimize societal costs and unwanted environmental impacts. Excessive losses of fertilizer nitrogen, whether added to agricultural, urban, or reclaimed soils, alter the economics of biomass production and increase the energy expenditures associated with industrial nitrogen fixation.

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Furthermore, increased utilization of industrially fixed nitrogen may have an environmental impact far beyond the localized site. Fertilizer production requires large quantities of fossil fuels. Utilization of these resources to produce plant nutrients not only decreases reserves of a nonrenewable commodity but also increases greenhouse gas production through its conversion to carbon dioxide and energy. (See van Groenigen et al. 2015 and Denk 2017 for more detailed presentations of the current status of our understanding of these processes.)

Denitrification defined: If nitrogen fixation is used as a reference for the initiation point of the nitrogen cycle, denitrification is its termination. Denitrification is the biological reduction of nitrogen oxides to nitrous oxide and/or dinitrogen. The process is catalyzed by a diverse group of microbes, including denitrifying and anammox bacteria and fungi that use nitrous oxide, nitrate, or nitrite as terminal electron acceptors in their respiratory processes associated with energy generation (see Long et al. 2013 and Strohm et al. 2007 for more detailed introductions to the growth and energy transformations involved). Since the electrons are more readily transferred to molecular oxygen compared to nitrogen oxides, denitrification can only occur under anoxic conditions. From this basic definition of denitrification, the complexity of controlling its occurrence in heterogeneous soils becomes evident. For denitrification to occur, the microorganisms must have an energy supply (usually an organic carbon source) and reside in an anaerobic soil microsite yet most soil systems are predominantly aerobic and the primary processes producing nitrate also have an obligatory requirement for molecular oxygen. That is, within the soil system itself, the primary source of the initial substance in the denitrification pathway, nitrate, is autotrophic nitrification, an obligatorily aerobic process.

These limited examples of the role of denitrification in controlling levels of nitrate occurring in soil and the availability of this primary plant nutrient within soil ecosystems underscore the need to develop an understanding of the basic properties of the process and the factors in the soil environment that control the rate of reduction of nitrate to dinitrogen. Thus, this chapter has the overall objective of elucidating the basic biological and biochemical properties of denitrification and determining the extent of its occurrence in terrestrial ecosystems, especially in intensively cultivated agricultural soils but also in wetland sites that are or could potentially bear the brunt of the influx of water from associated agricultural soils (e.g. Burgin et al. 2010; Rousk et al. 2018).

14.1 Pathways for Biological Reduction of Soil Nitrate

Soil fixed nitrogen resources may be conserved through both assimilatory and dissimilatory nitrate reductive processes. They may also be lost from the soil ecosystem by dissimilatory nitrate reduction. In this context, “conserved” refers to

retention within the soil ecosystem (generally as biomass, organic matter, or ammonium).

Assimilatory and dissimilatory nitrate reduction both involve the transfer of electrons to nitrogen oxides but they differ in the ultimate fate of the reduced nitrogen atom. With assimilatory reduction, nitrate is reduced to ammonium, which is subsequently incorporated into cellular biomass. Thus, with this process, the quantity of nitrogen reduced is proportional to cellular requirements for biomass production. In contrast, for dissimilatory nitrate reduction, the nitrogenous compounds are accepting electrons in support of cellular respiration. The final products (dinitrogen, nitrous oxide, or ammonium) are released from the cell and accumulate in the environment in concentrations far beyond those necessary for biomass synthesis. Note from the view of retention of fixed nitrogen within the soil ecosystem, dissimilatory processes can lead to losses (production of dinitrogen and nitrous oxide) or conservation (production of ammonium). These two fates of the nitrate reductive processes (dissimilatory vs assimilatory reduction) are characterized by commonalties of some of the intermediates and products (e.g. ammonium, nitrate). They are also distinguished by differences in the specific enzymes catalyzing the reductive reactions.

Since assimilatory reduction of nitrate is closely linked to biomass production, it is catalyzed by all soil inhabitants capable of using nitrate as a nitrogen source. Ammonium, the terminal product of the process, is incorporated into plant and microbial biomass. Also, although a portion of the cellular reducing power is transferred to the nitrogen, the cells rely upon other pathways as terminal electron acceptors (e.g. molecular oxygen, fermentative products).

Three commonly evaluated microbial processes are classed under the heading of dissimilatory nitrate reduction. These are distinguished by their respective products: (i) nitrite, (ii) ammonium, and (iii) nitrous oxide and dinitrogen (denitrification).

The first of these dissimilatory reductions involves the simple reduction of nitrate to nitrite. The general ability of many bacterial species to catalyze the process has led to the use of nitrate reduction to nitrite as a reliable taxonomic test for grouping bacterial isolates into genera and species. This reductive process is also significant in some *in situ* soil systems. In a soil site overlain by oxygen-bearing water, a nitrite and nitrate cycle that provides energy for nitrite oxidizers (see Figure 11.3) may develop. Under anoxic conditions, nitrate is reduced to nitrite by portions of the general soil bacterial population. This nitrite then diffuses from its source in the anoxic microsites to aerobic microsites where the autotrophic nitrite oxidizers utilize it in their energy-generating processes. Evidence for the occurrence of such interactions in native sites is the occurrence of an imbalance between ammonium and nitrite oxidizer populations in some soil ecosystems. The nitrite oxidizer populations are larger than would be expected to occur were ammonium oxidizers the sole provider of their energy resource, nitrite.

The second and third members of our list of dissimilatory nitrate reduction processes, dissimilatory ammonium production and denitrification, have several common characteristics. Both processes are coupled to organic matter decomposition (i.e. oxidation of fixed carbon compounds provides cellular energy and reducing power), the nitrogen oxides that are reduced serve as terminal electron acceptors, and growth yields of the bacteria are increased as a result of the energy provided by passage of an electron transport pathway that includes cytochromes (see Section 14.2 for discussion of the role of cytochromes in denitrification.).

Although not previously considered to be a major soil process, ammonium production from nitrate reduction has been shown to occur in soils and sediments (e.g. Buresh and Patrick 1978; Fazzolari et al. 1990; Koike and Hattori 1978; Smith and Zimmerman 1981). Dissimilatory ammonium production is catalyzed by a variety of enteric bacteria, *Bacillus* species, and *Clostridium* species in soil (e.g. Caskey and Tiedje 1980; Prakash and Sadana 1973; Rehr and Klemme 1989; Smith and Zimmerman 1981). As with denitrification, nitrous oxide has been found to be a product of metabolism of dissimilatory ammonium producers, but the nitrous oxide appears to be produced as a side reaction in these organisms (e.g. Smith et al. 1983).

14.2 Biochemical Properties of Denitrification

During biological denitrification, nitrate is transformed to dinitrogen by a series of reductive reactions as follows:

$$2HNO_3 \rightarrow 2HNO_2 \rightarrow \left[2NO\right] \rightarrow N_2O \rightarrow N_2$$

Nitric oxide is generally enclosed in brackets in depiction of this reaction sequence since it is not usually detected as a free intermediate. There is also some controversy over whether nitric oxide is a true intermediate in the process or whether its presence represents a side reaction (e.g. Betlach and Tiedje 1981; Garber and Hollocher 1981, 1982; Hochstein and Tomlinson 1988; Hollocher et al. 1980). In either case, the significant aspects of the reaction sequence from the soil microbiologist's viewpoint are that nitrate is reduced to nitrous oxide and dinitrogen with the possible transient accumulation of nitrite plus the occasionally detected transient accumulation of nitric oxide (e.g. Cady and Bartholomew 1960; Cooper and Smith 1963). As will be indicated below, a variable ratio – depending upon the soil chemical properties – of nitrous oxide to dinitrogen is produced.

14.2.1 Carbon and Energy Sources for Denitrifiers

The electrons for nitrogen oxide reduction are provided by oxidation of a variety of fixed carbon compounds. Most commonly, glucose is added to soil samples as a carbon

source in laboratory investigations of denitrification processes, but it must be remembered that a variety of organic compounds are catalyzed during this process. This array of oxidizable substrates includes many complex organic compounds, some of which are of concern from the view of soil contamination, such as acetone (Platen and Schink 1989); anthranilic acid (Braun and Gibson 1984); cresol (Bossert and Young 1986); and toluene (Evans et al. 1992). For a review of this topic, consult Evans and Fuchs (1988). Since the electrons for these oxidations are transferred through a chain of acceptors similar to the pathway leading ultimately to the reduction of molecular oxygen by aerobic heterotrophs, the growth yields of microbes oxidizing organic carbon substances while using nitrogen oxides as terminal electron acceptors are comparable with the two classes of acceptors (molecular oxygen and nitrogen oxides).

Using the traditional definition of anaerobic processes (i.e. energy-yielding catabolic reactions obligatorily occurring in the absence of molecular oxygen), dentrifiers could be considered to be facultative anaerobes. Historically, many soil microbiologists have not considered denitrification to be a true anaerobic process in that the primary overall difference between nitrate respiration and oxygen-based respiration is that in the former electron transport process, nitrate is merely replacing oxygen as the final electron acceptor. Denitrifiers are therefore generally thought to be aerobes. They use molecular oxygen as a terminal electron acceptor and do not ferment organic substances in the absence of molecular oxygen. They simply also possess cytochromes that can transfer electrons to nitrogen oxides. For example, in the process catalyzed by *Paracoccus denitrificans* terminal electron transfer to nitrate, nitrite and nitrous oxide are provided by cytochromes (e.g. Ballard and Ferguson 1988; Bolgiano et al. 1989) (Figure 14.1). If oxygen is present, different cytochromes are used for its reduction to water and dissimilatory nitrate reduction is inhibited (e.g. Hernandez and Rowe 1987).

14.2.2 Induction of Synthesis of Nitrogen Oxide Reductases

A further modification of cellular metabolism of the denitrifier growing in aerobic environs is that the requisite enzymes for denitrification are not synthesized or are produced in limited quantities. Nitrogen oxidase reductase synthesis is

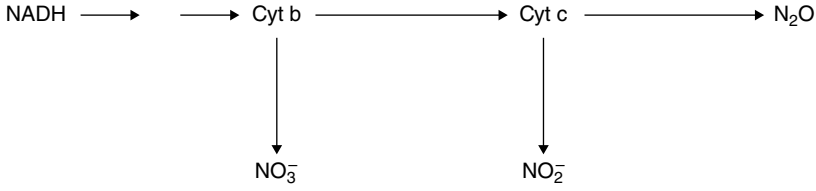

Figure 14.1 Summary of the electron transport pathway leading from oxidation of fixed carbon compounds to nitrate, nitrite, or nitrous oxide as terminal electron acceptors.

repressed by molecular oxygen. Once the oxygen is depleted in the microsite where the putative denitrifier resides, synthesis of these denitrifier enzymes is induced. Thus, development of a maximally functional denitrification system once anoxic conditions are imposed requires about 40 minutes to three hours. For some denitrifiers the time lapse necessary for synthesis of nitrogen oxide reductases is reduced by an induction of synthesis of the first enzyme in the pathway, nitrate reductase, as the available molecular oxygen supply is being depleted. Full induction of the pathway occurs with complete exhaustion of molecular oxygen resources. Thus, the microbe is able to respond to the absence of molecular oxygen by reducing nitrate to nitrite but it avoids synthesis of the full array of denitrification enzymes should anaerobiosis not be achieved. This differential synthesis of denitrification enzymes can result in a transient accumulation of nitrite.

14.3 Environmental Implications of Nitrous Oxide Formation

At one time, the transient accumulation of nitrous oxide in reaction vessels wherein biological denitrification was occurring was ignored or considered to be a product of the closed reaction system. It is now known that in native terrestrial environments, nitrous oxide is a common product of denitrification. For example, fluxes of nitrous oxide from soils have been shown to range from 7 to 165 kg N ha^{-1} yr^{-1} in drained histosols in South Florida (Terry et al. 1981a). Nitrous oxide evolution from mineral soil-based systems is usually less than reported from these drained organic soils. Annual losses from irrigated soils in California range from 19.6 to 41.8 kg N ha^{-1} (Ryden and Lund 1980). Lesser yields have been detected from cropped soils (Benckiser et al. 1996; Goodroad et al. 1984; Mosier and Hutchinson 1981) and prairie soils (Mosier et al. 1981). Intensity and nature of fertilization used in cropping systems also may affect the quantity of nitrous oxide produced (Loro et al. 1997; McTaggart et al. 1997; Mulvaney et al. 1997). Noncropped, native ecosystems also produce more limited quantities of nitrous oxide during plant growth seasons (Goodroad and Keeney 1984).

These data show that nitrous oxide production by soil processes is highly variable. Soil properties controlling the quantity of this product by soil microbes include soil redox potential (Letey et al. 1981; Smith et al. 1983), moisture tension (Davidson and Swank 1986; Freney et al. 1979; Regina et al. 1996; Terry et al. 1981b), nitrate and oxygen concentrations (Blackmer and Bremner 1978, 1979; Firestone et al. 1979, 1980), and the time of day that the measurements are taken (Blackmer et al. 1982). Generally, the proportion of gaseous nitrogen products composed of nitrous oxide increases with increasing soil acidity, reduced soil temperature, and

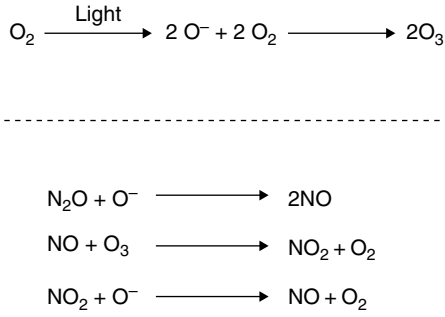

Figure 14.2 Reaction sequence leading to ozone formation and the role of nitrous oxide in its destruction.

augmented soil nitrate levels, but the exact ratio of nitrous oxide to dinitrogen for a specific soil system varies with the combination of the soil chemical and physical properties existent therein and is therefore not reliably predicted.

Understanding the variation of nitrous oxide in soil ecosystems has greater importance than simply allowing a better comprehension of soil nitrogen transformations. Nitrous oxide is an ozone-depleting greenhouse gas. The reactions involved in this process are presented in Figure 14.2. It must be noted that not all of the nitrous oxide evolving from the soil surface results from activity of denitrifiers. A variety of soil heterotrophs as well as autotrophic nitrifiers produce this gas as a metabolic product or by-product (e.g. Blackmer et al. 1980; Bleakley and Tiedje 1982; Bollag and Tung 1972; Davidson et al. 1986; Smith and Zimmerman 1981). Therefore the soil properties affecting nitrous oxide fluxes do not correlate only with those favoring biological denitrification. Complexity in interpreting the data is added by the fact that nitrous oxide is also produced by chemodenitrification as well as the various biological sources (see review by Bremner 1997 and examples of quantification of the processes in the field by Heinrich and Haselwandter 1997 and Nielsen et al. 1996).

14.4 Microbiology of Denitrification

14.4.1 Assessment of Soil Denitrifier Populations

The population density of denitrifiers in soil frequently has been estimated through use of most probable number (MPN) procedures (e.g. Focht and Joseph 1973; Volz 1977). These methods are based on detection of the conversion of nitrate or nitrite to gaseous end-products in liquid culture media (Figure 14.3). MPN methods are inherently imprecise (see Chapter 2 for a discussion of the limitations of MPN

Quantification of Denitrifiers

1. Prepare a serial dilution of a fresh soil sample [eg., 10^{-1}, 10^{-2}... 10^{-7}]
2. Inoculate 5 tubes containing 9 mL of nitrate broth with 1.0 mL of dilution.
3. Incubate the inoculated media at 30°C for two weeks.
4. Quantify residual nitrate in tubes. Presence of any nitrate = –.
5. Calculate MPN using standard tables.

Figure 14.3 Outline of a most probable number procedure for estimating denitrifier populations in soil. *Source:* Adapted from Focht and Joseph (1973).

procedures). More accurate and sensitive methods for enumeration of these organisms are now available as the characteristics of the genome encoding denitrification and the similarities in these gene sequences between the various denitrifiers present in soil samples are elucidated, thereby enabling the utilization of polymerase chain reaction (PCR) procedures for the quantification of denitrifiers in soil (e.g. Linne von Berg and Bothe 1992; Ye et al. 1993).

Denitrifier population density data do provide an indication of the number of organisms present in a soil ecosystem that are capable of catalyzing the process. Unfortunately, in most soils, population densities of denitrifiers do not correlate with denitrification rates. Soil microbial communities commonly contain several million denitrifiers per gram dry soil. Because of the fact that the respiration of these organisms is not limited solely to nitrate reduction (they can also reduce molecular oxygen), their presence in the system only indicates a potential for denitrification should conditions favor its occurrence. The denitrifier populations occurring in any particular soil with an oxygen-containing atmosphere may have developed through oxygen-based respiration.

14.4.2 General Traits of Denitrifiers

Denitrifiers are a biochemically and taxonomically diverse group of bacteria. Although some denitrifiers are chemoautotrophs (e.g. using hydrogen or reduced sulfur compounds as energy sources) and others are photoautotrophs, most of these organisms generally derive their energy from oxidation of fixed carbon substrates, including single carbon compounds. The primary substrate and end-product of the process are nitrate and dinitrogen, but some denitrifiers can only reduce nitrite (nitrite-dependent denitrifiers) whereas others lack nitrous oxide reductase, thereby producing nitrous oxide as the terminal product. The variety of metabolic types of bacteria capable of denitrifying defies any effort to group these organisms into one or a few bacterial genera.

Further difficulty with evaluation of the occurrence of the "denitrifier trait" in classical literature results from the fact that many organisms classified historically as denitrifiers are not really denitrifiers. Care must be exercised in classing organisms as

denitrifiers based strictly on the conversion of nitrate or nitrite to nitrous oxide or dinitrogen independent of the extent of the reaction. Many bacterial strains are capable of reducing nitrate and of producing *limited* quantities of dinitrogen or nitrous oxide. Although some of these organisms have been termed denitrifiers in the past, these marginal nitrate reducers are not true denitrifiers. To be a true denitrifier, a microbe must meet the following criteria.

- At least 80% of the nitrate or nitrite reduced by the bacterium must be converted to dinitrogen and nitrous oxide.
- There must be an increased growth yield due to the reduction of nitrate, nitrite, or nitrous oxide. This trait is the primary requirement for classing an organism as a denitrifier in that it shows that nitrogen oxide reduction is a dissimilatory process.
- The conversion of nitrate to nitrous oxide and dinitrogen must occur at a high rate. That is, the process must be central to cellular intermediary metabolism, not just a side reaction providing a minor pathway for electron transport.
- The presence of cytochrome cd or dissimilatory nitrite reductase should be demonstrable in the microbial cells.

14.4.3 Generic Identity of Denitrifiers

Many of the denitrifiers isolated from soils and waters are members of the genera *Pseudomonas* or *Alcaligenes*, but a large number of other bacterial genera contain strains of denitrifiers (e.g. Gamble et al. 1977). As with diazotrophs, denitrification is not a sufficiently definitive trait to allow separation of all denitrifiers into one or a limited number of genera. Not only are the individual bacterial strains of denitrifiers metabolically distinct, but the properties of the enzymes associated with denitrification process itself are highly variable. For example, in a comparison of denitrification by *Pseudomonas stutzeri, Ps. aeruginosa,* and *Ps. denitrificans* it was found that the rates of anoxic growth of the organisms varied 1.5-fold, gas production varied over eightfold, and cell yield differed by threefold (Carlson and Ingraham 1983). The metabolic and taxonomic diversity of these organisms is exemplified by the following partial listing of some of the bacterial genera containing denitrifiers (for a more complete listing of denitrifiers, see Knowles 1981, 1982).

- The genus *Achromobacter* is cited as containing a variety of denitrifiers, including methane oxidizers and nitrite-dependent denitrifiers. (These organisms have now been grouped among the species of the genus *Alcaligenes*.)
- *Alcaligenes eutrophus*, an organism originally classified as *Hydrogenomonas eutrophus*, is capable of autotrophic growth using hydrogen as an energy source, carbon dioxide for carbon, and nitrate as a terminal electron acceptor (e.g. Pfitzner and Schlegel 1973). This bacterial species is a facultative autotroph since it also uses organic carbon as an energy source. Other common denitrifiers in the

genus *Alcaligenes* include *A. denitrificans* and *A. odorans*. Another facultative autotroph which oxidizes hydrogen for energy is *Ps. denitrificans*.

- Several nitrogen-fixing bacteria are also capable of denitrifying. These diazotrophs include some strains of *Azospirillum brasilense* (e.g. Chauret et al. 1992; Neyra et al. 1977) and some *Rhizobium* species (e.g. Chan et al. 1989; Coyne and Focht 1987; Daniel et al. 1980; van Berkum and Keyser 1985; Zoblotowicz et al. 1978)
- Some denitrifiers are also thermophilic (e.g. Hollocher and Kristjánsson 1992).
- Some strains of *Chromobacter* denitrify using nitrate or nitrite as terminal electron acceptors, reducing them to nitrous oxide and dinitrogen, whereas *Chromobacter violaceum* can only reduce nitrogen oxides to nitrous oxide (Bazylinski et al. 1986).
- *Halobacterium marismortui* is a halophilic denitrifier that was isolated from the Dead Sea.
- *Hyphomicrobium* strains denitrify with methanol as the primary energy source. The practical significance of understanding the environmental limitations to activity of these strains relates to the fact that methanol has been used as an additive to sewage effluent to provide the energy source necessary for maximization of denitrification rates for removal of nitrate from the effluent.
- The photosynthetic bacterium *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* is both a denitrifier and a diazotroph (Dunstan et al. 1982).
- *Thiobacillus denitrificans* is an example of a sulfur oxidizing chemoautotroph which grows in the absence of oxygen with nitrate as a terminal electron acceptor. The energy sources for this organism include sulfide, elemental sulfur, and thiosulfate. *T. denitrificans* can also use molecular oxygen as a terminal electron acceptor.

14.5 Quantification of Nitrogen Losses from an Ecosystem via Denitrification

Historically, the most commonly used techniques for assessing nitrogen fixation have involved (i) determination of nitrogen balances in the ecosystem and (ii) quantification of distribution and movement of ^{15}N-labeled fixed nitrogen through soil reservoirs. More commonly used laboratory-based assays of denitrification are (i) assessment of disappearance of nitrogen oxides from soil slurries and (ii) use of acetylene inhibition of nitrous oxide reductase. The principles, advantages, and deficiencies of each of these techniques will be assessed herein.

14.5.1 Nitrogen Balance Studies

As was discussed previously in relation to quantification of nitrogen fixation in field sites, assessment of changes in nitrogen distribution among the various soil reservoirs as a means of quantifying process rates appears to be conceptually

simple and logically attractive. For application of nitrogen balance methods to quantification of denitrification, the amount of nitrogen denitrified is concluded to be the unaccountable or missing nitrogen. The quantity of nitrogen denitrified is that preexisting in the system plus any inputs (e.g. nitrogen fixation, fertilizer inputs) minus known losses (e.g. nitrogen in crops, nitrogen leached, ammonium volatilized). The procedure is also called the difference method because all nitrogen occurring in the system is subtracted from the sum of that previously occurring therein plus any amendments.

Use of the nitrogen balance method for estimation of denitrification processes in soils is predicated upon three assumptions.

- All of the organic and fixed nitrogen compounds contained in soil can be accurately quantified.
- The assay procedures available for quantifying soil nitrogen compounds are sufficiently sensitive to allow for detection of small changes in the various soil nitrogen compounds. Furthermore, it is assumed that the soil, water, and air samples analyzed are representative of the ecosystem and that no changes in the nitrogen levels existing in these samples at the site occurred between collection and analysis.
- The sole means of loss of gaseous nitrogen from soil is denitrification.

Unfortunately, none of these assumptions is valid. Reasonably sensitive assay procedures are available to assay soil organic and inorganic nitrogen pools. Their application to soil nitrogen cycle analysis is limited by the fact that changes in soil nitrogen reserves are commonly less than the precision of the assay method. Further problems with the direct quantification of nitrogen species in a particular soil site result from the fact that soil is an open system. That means water-soluble substances such as nitrate, nitrite, and simple organic nitrogenous compounds move freely into and out of the portion of the ecosystem of interest. Inability to accurately account for all fixed nitrogen inputs and losses from the soil site results in appreciable statistical variability in nitrogen balance data. This variation also results in invalidation of the second assumption.

Probably the biggest difficulty with application of nitrogen balance studies to assessment of denitrification is the third assumption (all nitrogen volatilization results from denitrification). There are at least three major routes of nitrogen volatilization from soil. Nitrogen may be lost through:

- nonbiological losses of ammonia (ammonia volatilization)
- chemical decomposition of nitrite
- denitrification as dinitrogen and nitrous oxide.

Ammonia volatilization can be a major route for loss of fixed nitrogen from soil. Up to 25% of the ammonia applied to soil as fertilizers or formed microbiologically may be lost as ammonia. Fortunately, for most soil systems, this process of nitrogen

volatilization is a minor concern since ammonia losses are insignificant below pH 7.0. (Recall that at acidic pHs the equilibrium between ammonium and ammonia is shifted so that ammonium predominates.) Ammonia volatilization is particularly apparent in the use of farmyard manure and at times in association with fertilization with urea. Major environmental concern with this process is linked more to the migration of soil fixed nitrogen to nearby bodies of water than with its interference with quantification of denitrification.

Nitrite decomposition (chemodenitrification) is rarely a major route of exodus of fixed nitrogen from soils. The most commonly documented situation for accumulation of meaningful nitrite concentrations is linked to alkaline soil conditions where nitrite accumulates due to ammonia toxicity to *Nitrobacter* spp. This phenomenon occurs in soils fertilized with anhydrous ammonia. In this process, nitrite reacts chemically with soil organic matter to produce dinitrogen and nitrous oxide plus some nitric oxide. In the absence of molecular oxygen, the nitric oxide may be evolved, but it is generally oxidized to nitrogen dioxide as soon as it is exposed to molecular oxygen. Although this reaction can occur in soil, nitrite will more commonly be biologically denitrified in soil. Thus, the relative importance of the three pathways of nitrogen volatilization from soils may be ranked, with denitrification being the major means of nitrogen loss, ammonia volatilization being ranked a distant second, and nitrite decomposition occurring only as a minor contributor.

The uncertainties associated with the use of nitrogen balance procedures for estimation of soil nitrogen losses due to denitrification were exemplified by data from a study in which this procedure was compared to results from a parallel quantification of nitrogen dynamics using ^{15}N to trace changes in soil nitrogen reservoirs (Rolston et al. 1979). In the more moist soils studied, actual denitrification losses were generally 0–30 kg N ha^{-1} less than amounts calculated from the nitrogen balance procedures. These differences ranged from 12 to 65 kg N ha^{-1} for drier soils. The authors concluded that with their soils, the uncertainties associated with the nitrogen balance procedure resulted primarily from large sampling variability in nitrogen leaching results (although variability of soil residual nitrate and organic nitrogen was also large). These results further emphasize the conclusion that even in soils where nitrogen volatilization due to chemical processes is minimal, the accuracy of the nitrogen balance procedure is limited due to difficulties in precisely accounting for all soil nitrogen forms present and leaving the soil system.

14.5.2 Use of Nitrogen Isotopes to Trace Soil Nitrogen Transformations

Although the short-lived radioactive nitrogen isotope (^{13}N) has been used to quantify denitrification processes (Firestone et al. 1979; Hollocher et al. 1980; Spier et al. 1995a, b; Tiedje et al. 1979), due to the short half-life of the radioactive isotope, the

heavy isotope of nitrogen (^{15}N) is more amenable to long-term quantification of denitrification reactions in soils. For these studies, ^{15}N-labeled fixed nitrogen sources (organic or inorganic) are amended to field soils or incubated soil samples (greenhouse or laboratory) and the ^{15}N-labeled dinitrogen or nitrous oxide quantified.

As with the evaluation of biological nitrogen fixation, implementation of this procedure is predicated on a series of assumptions, the most significant being (i) the denitrifier populations do not discriminate between nitrogen isotopes and (ii) the soil-amended ^{15}N-labeled fixed nitrogen is homogeneously mixed with native soil nitrogen resources and is thus equally available to the denitrifier population. As was noted with biological denitrification, satisfaction of these assumptions is questionable.

Discrimination of nitrogen isotopes by denitrifiers has been demonstrated (e.g. Blackmer and Bremner 1977). Clear microbial capacity to discriminate between ^{14}N- and ^{15}N-labeled nitrate was observed by these workers. Significant nitrogen isotope effects were detected in both reduction of nitrate to nitrite and nitrite to gaseous end-products.

Furthermore, because of the heterogeneity of the soil system, uniform mixing of an external nitrogen supply with indigenously formed fixed nitrogen is difficult at best. Vanden Heuvel et al. (1988) found that the magnitude of this error increases with the range of ^{15}N enrichment between native soil N isotope distributions and amended nitrogen (for a constant mean ^{15}N enrichment) and it varies depending on the number of nitrate pools existent in the system. The error was not affected by overall nitrogen gas evolution rate or the ^{15}N enrichment of the amended fertilizer. The theoretical basis for this potential for overestimation and underestimation of nitrous oxide and dinitrogen evolution was documented through mathematical modeling by Boast et al. (1988).

Even though these cited studies demonstrate significant difficulties with utilization of ^{15}N to quantify losses of fixed nitrogen from soil ecosystems due to denitrification, its application to field, laboratory, and greenhouse studies is appropriate when the limitations are considered. The use of ^{15}N is appropriate for field experiments (especially for long-term studies as exemplified by Focht and Stolzy (1978)) when the limitations are considered during data interpretations. ^{15}N labeling of nitrogen species in greenhouse and laboratory incubation studies has also been particularly useful. In the latter situations, extensive disruption of soil structure during sample preparation is common and the conditions for denitrification are more controlled.

14.5.3 Soil Nitrogen Oxide Transformations

Since nitrate, nitrite, and nitrous oxide are readily extracted from soil and quantified, it is reasonable to consider that the rate of change in concentrations (native or amended) of these chemicals can serve as an indication of denitrification rates

in soils (e.g. Bremner and Shaw 1958a) in laboratory-incubated soil samples. This procedure is based on the assumption that all of the nitrate or nitrite lost from the system is denitrified. Except in systems where extensive dissimilatory ammonium production is occurring, alternative fates of the nitrogen oxide (generally nitrate) tend not to interfere with this analysis procedure. Clearly, some nitrate could be incorporated into microbial biomass if the assay is conducted over a timeframe sufficiently long for microbial growth to occur. Thus, in this situation, denitrification rates would be underestimated by the amount of nitrate assimilated by the microbial biomass. Since the assays are conducted under laboratory conditions, plant uptake and leaching are not important because plants are excluded from the system and the reaction vessel is a closed system.

Greater problems with the use of changes in nitrogen oxide levels as an assessment of denitrification kinetics in soil are derived from the fact that in most soil systems, nitrate concentrations are a few $\mu g\ g^{-1}$ soil at best. Generally, nitrite is not detected in native soil samples. Thus, soils are generally amended with exogenous nitrate or nitrite for a meaningful estimate of the capacity of the soil microbial community to denitrify nitrogen oxides to be attained. To assure that the totality of the denitrification enzymes is measured (that is, enzyme and not substrate is the limiting factor), enzyme saturating concentrations of nitrate or nitrous oxide are generally added to soil with this technique. Therefore, since these nitrogen oxide concentrations are artificially elevated above those normally occurring in the field, denitrification rates assessed using this procedure must be understood to be potential rates rather than actual field values. The potential values would only be equivalent to field denitrification rates in the unusual situations when field nitrate concentrations are sufficient to saturate denitrification enzymes present.

14.5.4 Acetylene Block Method for Assessing Denitrification Processes in Soil

Incubation of soil samples or amendment of the soil atmosphere *in situ* with 10^{-2}atm of acetylene inhibits the reduction of nitrous oxide to dinitrogen (Yoshinari and Knowles 1976; Yoshinari et al. 1977). Since commonly available gas chromatography procedures may be used to quantify nitrous oxide, this provides a sensitive method for measurement of denitrification enzymes. (See Figure 14.4 for an outline of the procedure.) The validity of this technique for determination of denitrification kinetics in soil slurries (e.g. Smith et al. 1978) and in field situations (e.g. soil cores [Parkin et al. 1985a] and direct field measurements [e.g. Aulakh et al. 1991; Mosier et al. 1986]) has been confirmed through the comparison of data derived from use of acetylene block methods with those from ^{15}N and ^{13}N enrichments. Acetylene reduction inhibition of nitrous oxide reduction is a quick and reliable procedure for estimation of *in situ* levels of

Acetylene Block Method for Estimating
Soil Denitrification Potential

1. Place 10 g soil sample into 60 mL serum bottle
2. Add water or KNO_3 solution to soil
3. Purge and evacuate bottles with Argon or Argon + 10% acetylene
4. Incubate for appropriate time interval with mixing
5. Assay headspace N_2O with gas chromatographic procedure

Figure 14.4 Outline of a general procedure using the acetylene block method for quantifying denitrification potential in soil samples incubated in the laboratory. Samples receiving nitrate amendment provide estimates of maximum denitrification potential of the sample. Incubation times may vary from one hour to several days, depending upon the experimental objectives.

denitrification enzymes in soil slurries, soil cores, and the field. The initially constant nitrous oxide evolution rate in soil slurries corresponds to preexisting denitrifier enzyme in the soil sample (Smith and Tiedje 1979a,b).

A problem of providing an inhibitory level of acetylene throughout the soil sample is most frequently encountered in field sites and, to some degree, soil column experiments. In the field situation and with soil columns, care must be taken to assure complete admixture of the acetylene gas with the soil atmosphere. This limitation to the assay procedure is usually not important when using soil slurries since the suspensions are agitated at sufficient rates to guarantee distribution of the inhibitory gas through out the mixture.

Acetylene is toxic to ammonium oxidizers (e.g. Berg et al. 1982). Thus, in long-term field or laboratory studies where indigenous nitrification is relied upon for the nitrate source for denitrifiers, denitrification would be reduced or precluded. Implementation of this procedure in the field or with long-term incubated soil columns requires either duplication of sampling sites so that assays would not be repeated at the same specific site receiving acetylene or sufficient time must be allowed between assays for recovery of the nitrifier population to occur.

Additional difficulties may be encountered in the field should the acetylene itself stimulate carbon mineralization, thereby augmenting nitrate reduction (Haider et al. 1983). Nitrate amendment to soil samples may stimulate both aerobic and anaerobic acetylene oxidation. Based on this observation, Haider et al. (1983) conclude that repeated application of acetylene to the same field plot should be avoided.

It must be noted that these complications are not universally associated with field implementation of the acetylene inhibition procedure. Ryden and Dawson (1982) found no difficulty with its repeated use on grassland soils. In conclusion, these data further underscore the necessity of conducting appropriate controls to assure that the impact of acetylene on the soil system is direct alteration of nitrous oxide evolution through inhibition of nitrous oxide reductase and not indirectly through alteration of the activity of microbial populations dissociated from nitrate reduction.

Two additional factors to be considered in regard to the use of acetylene to estimate denitrification potential in soil is the capacity to estimate nitrous oxide production from both autotrophic nitrifiers and denitrifiers in the same soil sample and the existence of potential difficulties due to enhanced nitric oxide decomposition in soil receiving acetylene. Since nitrifiers and denitrifiers are inhibited by different concentrations of acetylene, variations in both concentration of acetylene and duration of exposure (Kester et al. 1996) or concentration (Inubushi et al. 1996; Webster and Hopkins 1996) have proven valuable in estimating the quantity of nitrous oxide produced by each source in soils and sediments. (Alternatively, Stevens et al. 1997 used differential ^{15}N-labeling of nitrate and ammonium pools to distinguish the two nitrous oxide sources.)

An additional concern in situations where nitric oxide is a by-product of denitrification is the impact of acetylene on the decomposition of the nitric oxide. Increasing acetylene levels in soil have been shown to increase the decomposition of nitric oxide by as much as 5–557-fold (Bollmann and Conrad 1997a). The consequence of the augmented nitric oxide decomposition is an underestimation of the associated denitrification rate (Bollmann and Conrad 1997b).

14.6 Environmental Factors Controlling Denitrification Rates

The primary factors that have been shown to control denitrification processes in the field are (i) the nature and amount of organic matter available as energy sources to the denitrifiers, (ii) the soil nitrate concentration, (iii) the aeration/moisture status of the soil, (iv) soil pH, and (v) soil temperature. Each soil property and examples of their effect in native soil systems will be evaluated herein.

14.6.1 Nature and Amount of Organic Matter

Most denitrifiers in soil are heterotrophs. Thus, the primary energy source for the process is plant biomass in most soil ecosystems. Although colloidal organic matter is a primary soil component, amendment of soil with metabolizable carbon generally does stimulate denitrification (e.g. de Catanzaro and Beauchamp 1985; Stanford et al. 1975b). For such experiments, saturating concentrations of nitrate are usually amended to soil slurries with the carbon and energy source (frequently glucose) varied. The increase in denitrification rate due to the augmented energy source generally resembles Michaelis kinetics (e.g. Bowman and Focht 1974; Reddy et al. 1982). Bowman and Focht (1974) found a Michaelis constant of 500 μg glucose mL^{-1} when nitrate reduction was assessed in a Coachella fine

sand. Further evidence for carbon limitations of denitrification rates is derived from the observation that denitrification rates generally correlate with total organic carbon and available carbon levels in soil. Available carbon has commonly been estimated by measurement of biological oxygen demand of soil water extracts (e.g. Beauchamp et al. 1980; Burford and Bremner 1975; Katz et al. 1985; Stanford et al. 1975c). There are rare situations in field sites where carbon does not limit denitrification. This exception is usually associated with mineral soils receiving high organic matter inputs (e.g. sludge-amended soils).

The generally observed correlation of denitrification rates with available organic matter leads to the prediction that treatment of soil to increase availability of native soil organic matter should result in an increase in the denitrification rate. Therefore, soil manipulations that alter the accessibility of indigenous organic matter to microbes can also change the indigenous denitrification rate measured. Freezing (e.g. McGarity 1962) and air drying (e.g. Patten et al. 1980) of soil samples can augment the denitrification rate detected with the samples. Thus, even with the most careful handling of field soil samples, the possibility that the laboratory-derived results are an overestimation of field denitrification rates must be considered.

An interesting possibility based on the observed proportionality of denitrification rates to available fixed carbon substrates is that the denitrification rate may be elevated in the rhizosphere compared to nonrhizosphere soil. Root exudates can be predicted to provide the energy source for the denitrifiers and anoxic microsites would be anticipated to occur in the vicinity of the roots where denitrifiers can function. Addition of macerated roots to soil samples as well as invasion of soil by root tissue has been shown to stimulate denitrification (e.g. Garcia 1975; Prade and Trolldenier 1988). Others (Haider et al. 1985, 1987; Smith and Tiedje 1979b) did not observe a rhizosphere effect on denitrification. Since the denitrifiers and the plants could be competing for the same nitrate pool, it could be hypothesized that the reason for the disparate observations is that different nitrate concentrations were used in the studies but Tiedje et al. (1979) found that variation of soil nitrate concentrations did not alter the denitrification rate in their rhizosphere samples. No rhizosphere effect on denitrification rates was detected with high nitrate concentrations. A reduction of denitrification occurred in the presence of low nitrate. Since the reduced nitrate concentrations used in their studies better reflected the generally encountered field situations, it could be predicted that denitrification rates in the rhizosphere should be reduced over those of nonplant-impacted soils.

These data exemplify the complexity of the interactions of physical, chemical, and biological factors in controlling denitrification in the rhizosphere. Although there is a clear impact of fixed carbon concentration on activity of denitrifiers, their function is also delimited by nitrate concentrations (for which they would be competing with plants as well as other bacterial populations), and anoxic microsites.

14.6.2 Nitrate Concentration

Since denitrification is an enzymatically catalyzed process, the reaction rate of the process is anticipated to follow Michaelis–Menten kinetics. That is, the rate of nitrate reduction to dinitrogen and nitrous oxide should increase until a saturating concentration is reached. Once enzyme saturating concentrations of the substrate have been reached, two alternatives are generally observed. There can be no effect of the augmented substrate levels or the substances may become inhibitory. The latter situation is generally detected in study of denitrification. That is, high nitrate concentrations tend to be inhibitory to nitrogen oxide reductases.

Nitrate reduction in soil is generally found to be either a zero-order or first-order process. For example, McGarity (1961) found denitrification rate to be independent of nitrate concentration (60–472 $\mu g\, g^{-1}$) in some South Australian soils. Bowman and Focht (1974) observed first-order kinetics with a Coachella fine sand. The Michaelis constant for the latter study was 170 μg nitrate mL^{-1}. Murray et al. (1989) measured Michaelis constants for mixed denitrifier populations from agricultural soils of 1.8–13.7 μM nitrate. Combinations of kinetic relationships may also be observed. Reddy et al. (1978) measured both first-order and zero-order reaction kinetics with flooded soils. In their study, nitrate disappearance from the waters above the soil was zero order whereas within the soil matrix, nitrate consumption followed first-order kinetics. In this situation, a nonbiological parameter entered into the reaction kinetics – diffusion of nitrate from the surface waters into the sediment soils. Diffusion limited the quantities of nitrate available to the soil microbes so the available nitrate was reduced by the microbial community as quickly as it entered into their microsite. Myrold and Tiedje (1985) suggest limitations to diffusion of nitrate to denitrifiers in large soil aggregates.

At high concentrations, nitrate inhibits nitrous oxide reductase. This inhibition varies with soil pH and history of the soil. Greater effect of high nitrate is detected at low pH, whereas extended flooding of the soil relieves the inhibitory effect of nitrate on the reductase.

14.6.3 Aeration/Moisture

As was noted above when the biochemistry of denitrification was analyzed, oxygen represses the synthesis of the reductases associated with denitrification. As soil oxygen tensions are reduced, reduction of nitrate to nitrous oxide and dinitrogen increases (e.g. Allison et al. 1960; Cady and Bartholomew 1961). Because of the slow rate of diffusion of molecular oxygen in water compared to air, this effect of aeration on denitrification rates in soils is highly related to soil moisture. In general, denitrification is not detected below 60% water-holding capacity. Above this value, denitrification rate generally correlates with soil moisture.

The field implication of this observation is that it can be anticipated that the majority of nitrate is denitrified in soil under flooded conditions. Generally, low denitrification rates are observed continuously in a drained soil system, but maximal activity is associated with increases in moisture due to rainfall (Sexstone et al. 1985a). In the field as soil moisture increases, loss of nitrate through denitrifier activity increases (e.g. Kroeckel and Stolp 1988; Pilot and Patrick 1972). This sensitivity of denitrifiers to increasing soil moisture explains a significant amount of the augmented loss of nitrate from no-till soils due to denitrifier activity compared to that occurring in conventionally tilled agricultural systems (Aulakh et al. 1984a, b; Rice and Smith 1982). This increase in denitrification results from augmentation of the number of anaerobic microsites in the soil. That is, the low amount of denitrification occurring in drained soils is occurring in anaerobic microsites and is not the result of activity of molecular oxygen-resistant denitrifiers (Hojberg et al. 1994; Sexstone et al. 1985b). Anaerobiosis is not the sole factor controlling the denitrification rate in the aggregate in that this rate does not correlate with the size of the anaerobic zone. Other contributing parameters are nitrate diffusion rate into the anaerobic microsites and the rate of molecular oxygen consumption by the aerobic organisms growing on the surface of the aggregate.

14.6.4 pH

Although denitrification occurs within the pH range of approximately 3.9–9.0, maximum nitrogen oxide reduction occurs from pH 7.0 to 8.0. The rate decreases as the pH is lowered (e.g. Bremner and Shaw 1958b; Focht 1974; Waring and Gilliam 1983). Acidophilic or acid-tolerant denitrifier populations are apparently selected in soils with histories of low pH. Parkin et al. (1985b) noted two distinctly different pH optima with soils of pH 3.9 and 6.3 that approximated the native pH values of the soils. It should be noted that the acidic soil used in the latter study had a 20-year history of low pH which would have provided more than adequate time for development of an acid-resistant soil microbial population.

Because of the occurrence of chemical denitrification in extremely acidic soils (e.g. Bollag et al. 1973), assessment of denitrification in such soils is difficult. Fortunately, the major products of the chemical and biological processes differ. Nitric oxide and nitrogen dioxide predominate from chemical reduction of nitrite whereas nitrous oxide and dinitrogen are biologically produced (Bollag et al. 1973). Unfortunately, dinitrogen and nitrous oxide are also reported among the products of chemodenitrification (e.g. Bulla et al. 1970; Reuss and Smith 1965). When the two processes are separated significant biological reduction of nitrous oxides can be shown to occur. Muller et al. (1980) found biological denitrification rates varied from 0.12 to 53.8 $\mu g\, d^{-1}$ in a variety of low pH ($pH \geq 3.6$) spodosols and peats collected from southern Finland. Complete inhibition of the reduction of nitrous

oxide to dinitrogen was found in 99.3% of these soil samples. Similarly, Gilliam and Gambrell (1978) found that acidity (pH values as low as 4.5) was not a serious limitation to denitrification in Atlantic coastal plain soils (U.S.A). As with the previous study, nitrous oxide was a major product of denitrification in these soils (Weier and Gilliam 1986). Similarly, in a study of a silt loam soil with pH varying from 4.6 to 6.9, nitrous oxide made up 83% of the gaseous nitrogen products of denitrification at pH 4.6 and 5.4. At pH 6.9, dinitrogen was the predominant product.

Frequently, several soil physical or chemical properties can interact to retard denitrification under acidic conditions. For example, Dubey and Fox (1974) found that low pH combined with low soil organic matter precluded denitrification in humid tropical soils of Puerto Rico. Similarly, George and Antoine (1982) noted that the pH optimum varied with temperature in their studies of a salt marsh soil. In their study, nitrous oxide was produced only at low pH and nitrate concentrations.

14.6.5 Temperature

The minimum temperature for denitrification is generally associated with the occurrence of free water. Smid and Beauchamp (1976) extrapolated their data from study of denitrification in the A horizon of a Huron clay loam to suggest denitrification at or near 0 °C. Incubation of the same soil under anaerobic conditions resulted in a complete inhibition of denitrification at 5 °C with a gradual increase in the rate to 30 °C (Bailey and Beauchamp 1973). Jacobson and Alexander in a study of two soils maintained in an anaerobic atmosphere (Jacobson and Alexander 1980) found no nitrate-reducing activity at 1 °C. Nitrate was slowly reduced at 7 °C. As with the previous studies, the rate increased with increasing soil temperature. These data suggest a minimum temperature for denitrification approaching the freezing point for water with an apparent impact of the presence of molecular oxygen on the limiting temperature. The maximum temperature is approximately that value limiting biological activity in soil in general, 75 °C. The optimum temperature (60–70 °C) is well above the normally occurring soil temperature range. In the range of increasing temperature, the nitrate reduction rate increases at a rate approximating that of general soil biological processes. That is, a Q_{10} of approximately 2 describes the relationship between increasing temperature and reaction rate (e.g. Stanford et al. 1975a).

These data indicate that significant denitrification can be anticipated to occur in any soil ecosystem at temperatures where general biological activity occurs. The Q_{10} value of about 2 indicates that the process is reasonably sensitive to slight fluctuations in soil temperature. Thus, not only should a seasonal variation in this process be anticipated, but also a diurnal impact. That is, losses of fixed nitrogen as nitrous oxide and dinitrogen will necessarily vary with daily heating and cooling of the upper portions of the soil profile.

14.6.6 Interaction of Limitations to Denitrification in Soil Systems

A strict consideration of each of the soil properties listed above and their impact on denitrification processes provides a limited view of the variation of nitrate reduction in field sites. In reality, each soil property is sufficiently variable across the soil landscape to provide significant landscape-scale variation in denitrification rates. Landscape analysis should be incorporated into models of soil fixed nitrogen losses through denitrification since topography has a major impact on *in situ* denitrification rates (Ball et al. 1997; Pennock et al. 1992). In the Pennock et al. (1992) study, when the site was considered as a whole, variables that were most influential on nitrogen losses were volumetric water content and soil redox potential. In the level portions of the site, volumetric water content was most highly correlated with denitrification whereas on the shoulder and foot slopes, respiration and bulk density were the most influential. Parkin (1987) found that "hot spots" of denitrification activity were associated with pockets of particulate organic matter. Parsons et al. (1991) found coefficients of variation between 74% and 268% for spatial variability of nitrogen gas loss from agricultural soils of central Kentucky (USA). Increases in nitrate reduction activity related to increases in soil moisture and soil respiration. These data support the conclusion that for a complete understanding of denitrification kinetics in a soil system, a study of the heterogeneity of the system, particularly with regard to soil moisture, organic matter, and most likely to some degree nitrate distribution, must be developed.

14.7 Conclusions

An understanding of the nuances of the impact of soil properties on biological denitrification and management of this process to regulate soil nitrate loadings is essential for optimization of agricultural production as well as for minimization of the potential for soil and water pollution. This terminal stage in the nitrogen cycle (return of fixed nitrogen to atmospheric reservoirs) is catalyzed by a diverse group of soil bacteria that are capable of using light, mineral (inorganic), or organic compounds as energy sources. The only readily discernible commonalties among denitrifiers are (i) they are solely bacteria, (ii) they are capable of reducing nitrogen oxides in the absence of free oxygen, and (iii) these nitrogen oxides serve as terminal electron acceptors for bacterial respiration. Thus, the primary determinants of denitrification rates in the field are the availability of energy sources, nitrate, and the absence of molecular oxygen from the microenvironment of the bacteria. The diversity of the organisms involved leads to the prediction that if life is possible in the system, a denitrifier should exist which is capable of functioning

therein. This adaptability and ubiquity of denitrifiers supports the conclusion that denitrification is a process amenable to management for control of soil nitrate loadings.

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15

Fundamentals of the Sulfur, Phosphorus, and Mineral Cycles

Biogeochemical cycles can easily be grouped into two major categories based on the significance of soil minerals to nutrient availability. As previously noted, in soils not receiving anthropogenically fixed organic carbon and nitrogen, photosynthesis and nitrogen fixation rates are major controllers of biological productivity of the soils. Once carbon dioxide and dinitrogen are incorporated into living biomass, the bulk of the cycling of these nutrients involves their movement into and out of the soil organic matter pools. Biomass levels are controlled, at least in part, by dissolution of soil minerals.

The main objective of this chapter is to introduce the primary processes associated with solubilization of sulfur, phosphate, and metal nutrients and the incorporation of the mineral forms of these essential nutrients. Biological inputs into these nutrient cycles are emphasized.

15.1 Sulfur in the Soil Ecosystem

Sulfur atoms occur in soil in a wide variety of organic and inorganic compounds. Sulfur is found in humus, animal, plant and microbial biomass, sulfur-bearing minerals and a variety of water-soluble compounds. Volatile sulfur-containing compounds are found among the gases making up the soil atmosphere and ionic forms contribute to the salt content of interstitial water. The primary water-soluble ionic sulfur form in soils is sulfate. As a gas, sulfur atoms are detected in hydrogen sulfide, which may occur at significant concentrations in anoxic, waterlogged soils. Sulfur contributes to the soil particulate fraction in both soil organic matter and soil minerals. Examples of sulfur-bearing minerals include pyrite (FeS_2), occurring in igneous rocks, and gypsum ($CaSO_4.2H_2O$) and epsomite ($MgSO_4.7H_2O$).

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Quantities of organic and mineral sulfur in soil are extremely variable, ranging from as low as 0.002% in highly leached, weathered soils of humid regions to concentrations as high as 5% in calcareous and saline soils of arid and semiarid regions (Stevenson 1986). Much of the soil sulfur is derived from internal sources but external inputs, such as fertilizers, air pollutants (e.g. sulfur dioxide), acid mine drainage (sulfuric acid), and a variety of xenobiotic compounds (e.g. pesticides), may also be significant sulfur sources. Basically, essential pools of sulfur in soil include not only major components of living biomass (e.g. biotin, coenzyme A, thiamine, glutathione, cysteine, cystine, methionine) (Figure 15.1), but also inorganic sulfur-based energy sources (e.g. sulfide, S^{2-}, elemental sulfur S^0), as well as terminal electron acceptors supporting microbial metabolism (e.g. sulfate, SO_4^{2-}).

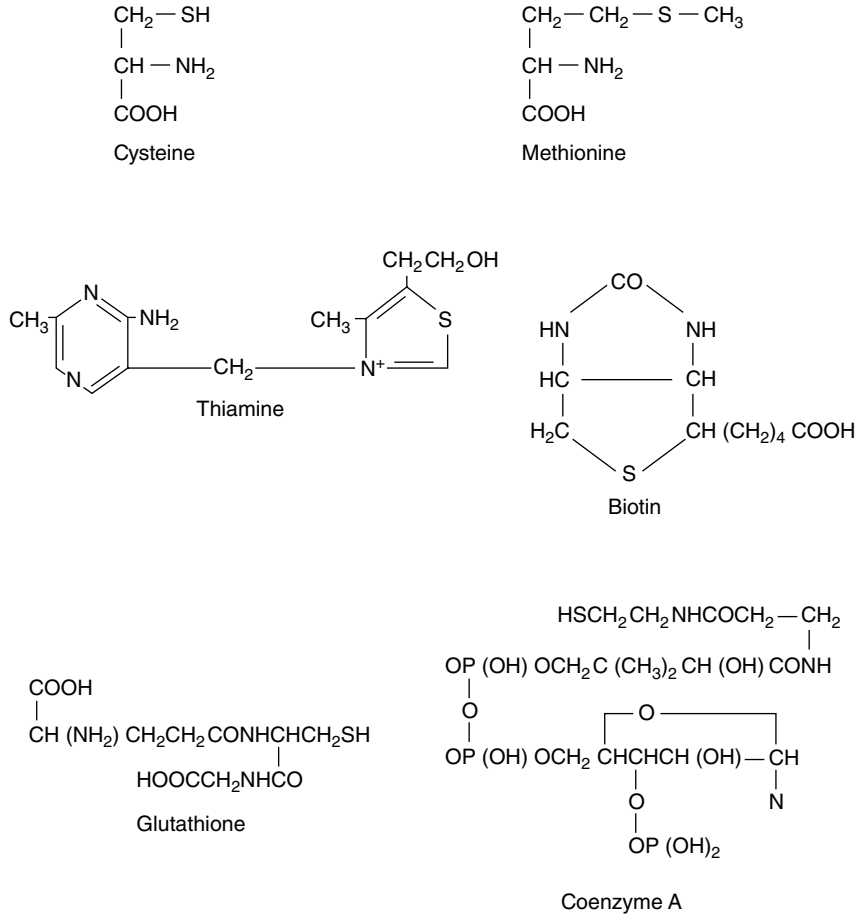

Figure 15.1 Examples of organic sulfur compounds found in soil.

A primary property of the sulfur atom contributing to its utility and versatility in biological systems is its wide range of commonly occurring oxidation states. Common valence states of sulfur relating to biological active molecules are −2 (e.g. in organic compounds as sulfhydrals and in mineral sulfides), 0 (elemental sulfur), and +6 (e.g. organosulfates and mineral sulfates). See Table 15.1 for further demonstration of the variation in valence of sulfur atoms in commonly occurring soil sulfur compounds. Microbes are the primary mediators for cycling the sulfur atom among these various oxidation states, although spontaneous chemical processes contribute to these transformations to a limited degree.

15.2 Biogeochemical Cycling of Sulfur in Soil

A generalized schematic of the sulfur cycle is depicted in Figure 15.2. As with those biogeochemical cycles previously evaluated, sulfur atoms are transferred between a variety of organic and inorganic reservoirs. Primary soil-resident sulfur pools are organic residues (plant, animal, and microbial biomass plus their decomposition products and the humic substances derived from them) and soil minerals. In contrast to the nitrogen cycle, except in limited situations (e.g. anthropogenically generated sulfur dioxide produced primarily by the burning of fossil fuels), atmospheric contributions of sulfur compounds to this biogeochemical cycle are limited. This diversity of organic and inorganic sulfur reservoirs suggests a complexity in sulfur cycling in soil not previously encountered in our study of carbon and nitrogen cycles. Sulfur cycling is controlled to a large degree by levels of interaction between soil mineral pools and living biomass in a manner unique to the microbial community (that is, inorganic sulfur serves as both a

Table 15.1 Oxidation state of major sulfur compounds in soil

Compound	Oxidation state
Organic S (R-SH)	−2
Sulfide (S^{2-})	−2
Elemental S (S^0)	0
Thiosulfate ($S_2O_3^{2-}$)	2+ (average/S)
Tetrathionate ($S_4O_6^{2-}$)	+2 (average/S)
Sulfur dioxide (SO_2)	+4
Sulfite (SO_3^{2-})	+4
Sulfate (SO_4^{2-})	+6

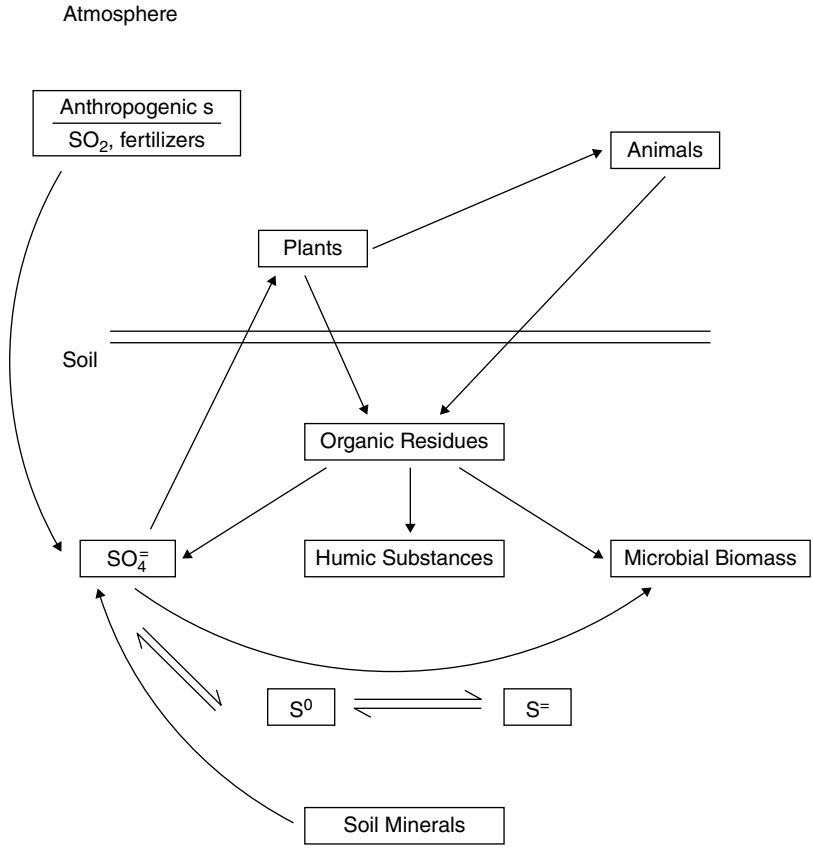

Figure 15.2 Major features of the sulfur cycle.

terminal electron accepter, e.g. metabolic reduction of sulfate to sulfide, and a microbial energy source, e.g. oxidation of sulfide to sulfate).

- *Cycling of sulfur between soil biomass reservoirs and water-soluble sulfate (assimilation and mineralization)*: Sulfate incorporated into biomass may be derived from mineralization of native soil organic matter as well as microbial, plant, and animal residues. Organic sulfur compounds contained in the decaying biomass are mineralized and incorporated into newly synthesized cells. Additionally, the microbial cells may also directly incorporate components of partially decayed biomass, including sulfur-bearing amino acids and vitamins, into their cellular structure, as exemplified by the mineralization of organic wastes (e.g. farmyard manure) in soil, where portions of the sulfur-bearing decay products of the organic matter are incorporated directly into growing plant and microbial biomass.

- *Complexing of sulfide and sulfate into soil minerals and their solubilization to biological available substances*: Some mineral sulfur may be lost from the above mineralization-assimilation cycle through transfer to water-insoluble reservoirs. Sulfide accumulation in soil minerals has been defined as *sulfidization* whereas the exposure of sulfide-bearing minerals to oxidizing conditions resulting in production of sulfate is *sulfurization*. These processes may involve dissolution or precipitation of the minerals through chemical processes (e.g. precipitation of sulfide by iron or solubilization of minerals under acidic conditions) as well as biological oxidation or reduction of the primary soil mineral components. This portion of the overall sulfur cycle is highlighted because it represents the formation of an inorganic sulfur pool with long-term implications for the geological portion of the soil biogeochemical cycle.

This model of the sulfur cycle emphasizes the primary sulfur resources in soil ecosystems. Examples of other sulfur-containing reservoirs that may be of significant environmental importance, at least on a localized basis, include (i) societal waste products incorporated into soil (e.g. sewage biosolids, composts, organic matter in household wastes, and fly ash from electric power production), (ii) agricultural chemicals (including pesticides), and (iii) air pollutants (e.g. products of fossil fuel combustion in electrical power generation and in automobile exhausts). These substances enter the soil sulfur cycle through reactions similar to those already depicted. For example, sulfur dioxide produced from burning fossil fuels enters the interstitial water where it is oxidized to sulfate. The anthropogenically produced organic sulfur is mineralized as described above for native biomass of the ecosystem.

The most obvious atmospheric contribution to the sulfur cycle is the previously mentioned sulfur dioxide, but it must be understood that particulate (dry deposition) sulfur inputs may also be significant on a localized basis. As with the sulfur dioxide, particulate sulfur inputs (primarily as elemental sulfur but also as various sulfates) contribute to soil acidity by increasing sulfate concentrations. An extreme example of the impact of deposition of elemental sulfur on an ecosystem is provided by studies of forest ecosystems in Alberta, Canada. These sites received large quantities of industrially produced sulfur. Kennedy et al. (1988) and Maynard et al. (1986) both report dramatic declines in aboveground vegetation quantity and diversity due to increasing soil sulfur loadings. Sulfur inputs reported by Maynard et al. (1986) ranging from 4100 to 51 400 $\mu g\ g^{-1}$ soil resulted in decreasing soil pH (4.4–2.4) and augmentation of the *Thiobacillus thiooxidans* populations. Visser and Parkinson (1989), in their study of forest soils receiving comparable elemental sulfur loadings, found a decline in microbial biomass that correlated with soil pH and a parallel reduction in the ability of the soil community to mineralize glucose. The environmental impact of the perturbation of the sulfur cycle resulting from deposition of unusually large quantities of elemental

sulfur is a logical outgrowth of the capacity of the native soil microbial population to use this sulfur substance as an energy source.

15.3 Biological Sulfur Oxidation

Chemoorganotrophic and chemolithotrophic bacteria derive their energy from oxidation of organic and mineral substrates. The large range of oxidation states of the sulfur atom (−2 to +6) make it a nearly ideal candidate as a primary energy source for resourceful soil microbes. In soil ecosystems, a variety of soil microbes have the capacity to derive their energy from oxidation of inorganic sulfur compounds. This exploitation of sulfur oxidation is a minor biogeochemical process in that terrestrial ecosystems are predominantly driven by heterotrophic processes. Sulfur oxidation can be an important energy source for some portions of the terrestrial ecosystem, such as exotic deep sea hydrothermal vents. Both sulfide and elemental sulfur are substrates in this energy-yielding process.

The diversity of the oxidation status of the sulfur atom allows for the concurrent oxidation and reduction of the same compound by the same microbe. In this *disproportionation* process, the sulfur atom is serving as both an electron donor and electron acceptor for the microorganism. Examples of the process are demonstrated by the disproportionation of thiosulfate and sulfite by *Desulfovibrio sulfodismutans*. Sulfate and sulfide are formed from the sulfur compounds by the following reactions:

$$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + H_2S$$

$$4SO_3^{2-} + 2H^+ \rightarrow 2SO_4^{2-} + H_2S$$

15.3.1 Microbiology of Sulfur Oxidation

Oxidation of sulfur-containing compounds, especially elemental sulfur, has a major impact on overall ecosystem function, primarily due to the fact that the terminal product of the reaction, sulfate, results in acidification of the soil. Elemental sulfur has long been used as a soil amendment when lowering of soil pH is required. Environmental degradation has been linked with excessive oxidation of sulfur, as can result from mining of sulfide-bearing ores (acid mine drainage) or atmospheric deposition of sulfur dioxide due to acid rain produced during energy recovery from fossil fuels. Thus, an understanding of the biological interactions involved with the oxidation of sulfur compounds and the nuances of environmental management of the process is essential for proper stewardship of soil resources as well as for reclamation of mismanaged systems.

15.3.1.1 The Thiobacilli

Although a diverse group of soil microbes are capable of oxidizing sulfur and sulfide for energy, members of the bacterial genus *Thiobacillus* are the primary mediators of this process in soil ecosystems. Some studies suggest that heterotrophic bacteria and fungi may also be of some importance in this biogeochemical process, but the significance of their contribution has yet to be elucidated.

The thiobacilli are chemoautotrophic or facultatively chemoautotrophic bacteria that are widely distributed in soil ecosystems. These generally motile, gram-negative bacteria are distinguished from other members of the soil community by their capacity to catalyze at least some portion of the oxidative continuum of sulfide, elemental sulfur, thiosulfate, and/or tetrathionate to sulfate. Among the energy substrates of the five most studied members of this genus (*T. thiooxidans, T. ferrooxidans, T. thioparus, T. novellus,* and *T. denitrificans*), elemental sulfur, thiosulfate, tetrathionate, and sulfide are the most commonly listed. It must be noted that each species of the genus *Thiobacillus* is capable of oxidizing a specific portion of the spectrum of oxidizable sulfur compounds occurring in soil. For example, not all species or strains within a given species are capable of oxidizing elemental sulfur. Furthermore, not all *Thiobacillus* species are limited to deriving their energy from oxidation of sulfur compounds; fixed carbon and ferrous iron may also be oxidized. The described species can be divided into three groups based on their sensitivity to soil pH and their capability to oxidize fixed carbon: neutrophilic chemoautotrophs, neutrophilic facultative autotrophs, and acidophiles (Kuenen et al. 1992). Of the most studied members of this genus, *T. novellus* is a facultative chemoautotroph and *T. ferrooxidans* recovers some energy from ferrous iron oxidation (Table 15.2).

These sulfur-oxidizing bacteria are further distinguished by the nature of their terminal electron acceptor and their resistance to acidic conditions. Resistance to extreme acidity by thiobacilli is particularly valuable in that they may produce sufficient sulfate to lower the soil pH to values between 1 and 3. Most members of the genus are obligate aerobes, although *T. denitrificans* can use oxygen or nitrate

Table 15.2 Metabolic properties of commonly studied *Thiobacillus* species

***Thiobacillus* species**	**Optimum pH**	**Energy substrates**	**Electron acceptors**
T. thiooxidans	2.0–3.5	S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$	O_2
T. ferrooxidans	2.0–3.5	S^0, $S_2O_3^{2-}$, Fe^{2+}	O_2
T. thioparus	Approx. 7.0	S^0, S^{2-}, $S_2O_3^{2-}$, $S_4O_6^{2-}$	O_2
T. novellus	Approx. 7.0	$S_2O_3^{2-}$, organic compounds	O_2
T. denitrificans	Approx. 7.0	S^0, S^{2-}, $S_2O_3^{2-}$, $S_4O_6^{2-}$	O_2, NO_3^-

as its terminal electron acceptor and *T. ferrooxidans* transfers electrons to ferric ion under anoxic conditions. A wide range of tolerance to acidity is characteristic of the genus. For example, *T. thiooxidans* and *T. ferrooxidans* have pH optima between 2.0 and 3.5. In contrast, *T. thioparus, T. denitrificans*, and *T. novellus* grow optimally at near neutral pH values (Table 15.2). For a more general discussion of the properties and function of *Thiobacillus* species, see Alexander (1977), Harrison (1984), Kuenen et al. (1992), Stevenson (1986) or Visniac and Santer (1957).

The kinetics of sulfate formation by these microbes can be exemplified by a list of the reactions involved in elemental sulfur, thiosulfate, and tetrathionate by *T. thiooxidans*:

$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$$

$$Na_2S_2O_3 + 2O_2 + H_2O \rightarrow H_2SO_4 + Na_2SO_4$$

$$2Na_2S_4O_6 + 7O_2 + 6H_2O \rightarrow 6H_2SO_4 + 2Na_2SO_4$$

T. ferrooxidans oxidizes elemental sulfur and thiosulfate by reactions similar to those listed above for *T. thiooxidans*, but this oxidation is combined with the oxidation of ferrous to ferric iron as follows:

$$12FeSO_4 + 3O_2 + 6H_2O \rightarrow 4Fe_2\left(SO_4\right)_3 + 4Fe\left(OH\right)_3$$

This species also contains enzymes capable of reducing ferric iron (Fe^{3+}) (Sugio et al. 1987), tetravalent manganese (Mn^{4+}) (Sugio et al. 1988a), cupric ion (Cu^{2+}) (Sugio et al. 1990), and molybdic ion (Mo^{6+}) (Sugio et al. 1988b). The environmental significance of the latter processes has not been elucidated.

The oxidative process for *T. denitrificans* is coupled to the reduction of nitrate as follows:

$$5S + 6KNO_3 + 2H_2O \rightarrow K_2SO_4 + 4KHSO_4 + 3N_2$$

The environmental significance of this process is that it provides a mechanism for sulfur oxidation in anoxic environments. Alternatively, ferric ion may also serve as a terminal electron acceptor for sulfur oxidizing *T. ferrooxidans* growing in the absence of molecular oxygen. Pronk et al. (1992) have shown a linear relationship between ferrous iron accumulation and cell density in *T. ferrooxidans* reducing ferric iron and oxidizing elemental sulfur. Suzuki et al. (1990) have also shown that several strains of *T. ferrooxidans* are capable of oxidizing elemental sulfur under anoxic conditions when using ferric ion as a terminal electron acceptor. Some of the strains oxidized the sulfur with ferric ion as the electron acceptor at rates comparable to those of the aerobic process, but with others, sulfur oxidation occurred at a rate 50% or more lower than that occurring in the presence of oxygen.

A variety of other metal-mobilizing, sulfur-oxidizing *Thiobacillus* species have been evaluated, e.g. *T. acidophilus* (Meulenbert et al. 1992), *T. prosperus* (Huber and Stetter 1989), *T. versutus* (Beffa et al. 1991) and *T. cuprinus* (Huber and Stetter 1990). Other autotrophic bacteria that catalyze sulfur oxidation under aquatic or more specialized conditions include phototrophic bacteria (Brune 1989) and *Sulfolobus* species that function in hot springs (Alexander 1977).

15.3.1.2 Heterotrophic Sulfur Oxidizers

Several heterotrophic microbes also catalyze sulfur oxidation. *Aspergillus niger*, *Mucor flavus*, and *Trichoderma harzianum*, for example, oxidize elemental sulfur (Grayston et al. 1986). These fungi produce substantial amounts of sulfate from sulfur oxidation with thiosulfate (*A. niger*) or thiosulfate and tetrathionate (*M. flavus*) accumulating as intermediates. *Aureobasidium pullulans* (de Bary) Arnaud, isolated from sycamore phylloplane previously exposed to atmospheric pollution, oxidized elemental sulfur to dithionite, tetrathionate, and sulfate (Killham et al. 1981). Similar products are produced by yeast isolates of the genus *Rhodotorula* (Kurek 1979, 1985) and bacteria (Pepper and Miller 1978) and actinomycete (Yagi et al. 1971) isolates. These heterotrophic microorganisms utilize carbonaceous substrates for their carbon and energy source and generally accumulate oxidized sulfur compounds after the period of active growth. Thus, their role in sulfur oxidation in native soil ecosystems is questioned.

Indirect evidence suggests that there is at least some role for the heterotrophs in elemental sulfur oxidation in soil. Lawrence et al. (1988) evaluated the effect of elemental sulfur fertilization on a variety of measures of sulfur oxidation in two Gray luvisolic soils from Canada. In one soil, increases in *Thiobacillus* populations were induced by elemental sulfur amendment whereas in the other soil no change in these populations was detected. Heterotrophic sulfur oxidizers were the most abundant sulfur oxidizers in both soils. In a related study (Lawrence and Germida 1988), elemental sulfur oxidation in 28 Canadian soils correlated linearly with soil microbial biomass carbon ($r = 0.68$, $p < 0.01$) and soil respiration ($r = 0.88$, $p < 0.01$). Further evidence implicating a heterotrophic contribution to the sulfur oxidation included (i) augmentation of heterotrophic biomass through glucose amendment increased sulfur oxidation rates and (ii) inhibition of general heterotrophic microbial activity reduced sulfur oxidation rates.

In contrast, Lee et al. (1987), in their study of 48 New Zealand soils, found that elemental sulfur amendment resulted in increases of thiobacilli populations with no change or a decline in heterotrophic sulfur-oxidizing populations. These reports suggest that environmental selection is likely of great significance in determining the dominant populations involved in sulfur oxidation in soil ecosystems. Of primary importance in considering the impact of autotrophic vs heterotrophic populations in modified sulfur cement oxidation is the requirement of

oxidizable carbon substrates for function of the heterotrophs. These substrates are concentrated in surface horizons of the soil profile and are limiting in subsoils (Tate 1987).

15.3.2 Environmental Conditions Affecting Sulfur Oxidation

Although sulfur-oxidizing microbes are considered to be ubiquitous, populations of autotrophic thiobacilli can be essentially undetectable in some soils. Increases in populations can result from sulfur amendment to the soil. For example, thiobacilli population augmentation by sulfur amendment has been demonstrated with several soils from Kansas (Attoe and Olson 1966), Canada (Janzen and Bettany 1987a), and New Zealand (Lee et al. 1988a). The increased sulfur-oxidizing population induced by sulfur amendments can result in increased sulfur oxidation rates in subsequent growing seasons (Lee et al. 1988b). These observations relate specifically to *Thiobacillus* populations. Since, as indicated above, a variety of heterotrophic microbes are also capable of oxidizing sulfur in soil, it is reasonable to conclude that sulfur oxidation is not precluded in soils due to nonexistence of requisite microbes. Significant autotrophic or heterotrophic populations can be anticipated to occur in any biologically active soil. The rate may be limited due to initially low population densities but the numbers of autotrophic microbes should increase following amendment with sulfur substrates. Therefore, it can be concluded that since the requisite sulfur oxidizers can be assumed to be present in any given soil ecosystem, oxidation of sulfur is dependent primarily upon occurrence of physical and chemical conditions conducive to the function of these microbes.

Basically, for a functional soil microbial community to develop there must be both an energy source (an oxidizable substrate) and an electron acceptor. For most living organisms, these are a fixed carbon supply, such as simple sugars, carbohydrates or amino acids, and oxygen. With elemental sulfur oxidation, the sulfur can serve as the electron source, i.e. the oxidizable substrate, and oxygen is the electron acceptor. As noted above, one species of elemental sulfur oxidizer, *T. denitrificans*, that is capable of oxidizing sulfur under anoxic conditions has been isolated. Cytochromes are used in the electron transport system and the energy yield approaches that gained when oxygen serves as the electron acceptor. Although some strains of *T. denitrificans* have been shown to be unable to oxidize extracellularly available elemental sulfur anaerobically (Schedel and Trueper 1980), others function quite efficiently with nitrate as an electron acceptor and elemental sulfur as the energy source. For example, a *T. denitrificans*-based system is sufficiently efficient that it has been proposed as a means of removing excess nitrate from waste water. Elemental sulfur serves as a cheap source of energy for the denitrification process (Batchelor and Lawrence 1978). High percentages of denitrification (over 95%) have been demonstrated using this process (Hasimoto et al. 1987).

The use of elemental sulfur as an energy source in soil is limited by the capacity of the microorganism to interact with the substrate. Physical attachment of thiobacilli to sulfur particles is required for enzymatic oxidation of sulfur. This interaction of the microbes with sulfur particles can be the rate-limiting factor determining the sulfur oxidation rate. This was shown clearly 80 years ago in a study of *T. oxidans* conducted by Vogler and Umbreit (1941). Additionally, Espejo and Romero (1987) demonstrated that for *T. ferrooxidans* only bacteria attached to the sulfur particle were capable of growth. No replication occurred once the bacteria were detached from the sulfur particle. Growth of the microbes was not uniform on the sulfur particle surface but rather it occurred in distortions present on the particle surface. Staining of sulfur prills with crystal violet revealed that even after more than four months incubation, intact regions remained on the substrate surface whereas destruction of the surface had occurred quite deeply at other locations on the prill.

Strains of *T. ferrooxidans* also have a more versatile energy recovery capability. When attached to sulfur particles, sulfur oxidation is the primary energy-yielding mechanism. Microbes that become detached from the sulfur particles can survive through the oxidation of ferrous ion to ferric ion. The sulfur oxidation rate was not affected by the presence of ferrous ion in the growth medium (Espejo et al. 1988). For *T. albertis* specific cellular adhesions, bacterial glycocalyx, are produced to facilitate the interaction of the thiobacilli cells with the sulfur particle (Bryant et al. 1984). Elemental sulfur oxidation by the latter *Thiobacillus* species is proportional to particle size, surface area per unit weight and the crystal microstructure of the elemental sulfur (Laishley et al. 1986).

Since the elemental sulfur particles do not dissolve in water and direct contact of the microbial cell with the sulfur particle is necessary for oxidation to proceed, it can be concluded that surface area of the sulfur particles controls their oxidation rate in soils. This has been demonstrated repeatedly in a wide variety of soils over the last several decades (e.g. see Bertramson et al. 1950; Frederick and Starkey 1948; Janzen and Bettany 1987b; Laishley et al. 1986; Lee et al. 1988a, b; McCaskill and Blair 1987; Nor and Tabatabai 1977; Watkinson 1989). For each of these studies, a size distribution of sulfur particles was added to soil and sulfate concentration or pH changes due to sulfur oxidation were quantified as a function of particle size. In each case, rapid oxidation was detected with small particles and limited reaction of the larger particles occurred. For example, Lee et al. (1988a) found that after 340 days, 80–90% of elemental sulfur particles less than 0.15 mm diameter were oxidized as opposed to only 24–55% of the particles greater than 0.15 mm diameter. McCaskill and Blair (1987) found that with mixtures of particle sizes, a biphasic oxidation curve could be detected. A reduction in conversion rate occurred once the 0.1 and 0.2 mm diameter particles were exhausted. Frederick and Starkey (1948) evaluated particles of sulfur in pipe sealing mixtures. They

found that over a three-week incubation period, sulfur oxidation was essentially nondetectable with particle sizes greater than about 3 mm mean diameter.

Along with availability of sulfur-oxidizing microbes in soil and their growth substrates, sulfur oxidation is controlled by soil moisture, pH, and temperature. Because of the physical stability of elemental sulfur particles in soil, the impact of these factors on sulfur oxidation relates to their effect on the microbes catalyzing the reactions.

In soil, the rates of oxidation of sulfur-containing compounds and elemental sulfur tend to increase with increasing soil moisture, reach an optimum level, and then rapidly decline (e.g. Attoe and Olson 1966; Janzen and Bettany 1987b; Lettl et al. 1981; Moser and Olson 1953). With slight variations dependent upon soil texture, optimum soil moisture levels are near field capacity.

Complex interactions between soil moisture, soil texture, and microbial populations alter elemental sulfur oxidation rates (Janzen and Bettany 1987a, b; Watkinson 1989). In one study of soils with limiting oxidizer population densities (as indicted by responses to inoculation), elemental S conversion rate was not affected by clay contents ranging from 9% to 52% (McCaskill and Blair 1987). This contrasts with data from Janzen and Bettany (1987b) who found that with 40 diverse Canadian soils, the elemental sulfur oxidation rate was negatively correlated with soil clay contents. Watkinson (1989) found that the improved soil porosity resulting from amendment of phosphate rock to mineral soils augmented elemental sulfur oxidation. It can be concluded that the prevailing controller of elemental sulfur oxidation in soils of varying moisture and texture is the availability of the obligatory electron acceptor, molecular oxygen.

Although elemental sulfur is used to reduce soil pH, its oxidation rate actually generally increases when pH is raised. Both Attoe and Olson (1966) and Janzen and Bettany (1987b) found a positive correlation of elemental sulfur oxidation rate and soil pH in a wide variety of soils. Further, Lettl et al. (1981) noted that oxidation of elemental sulfur was stimulated by amendment of soil with calcium carbonate.

Elemental sulfur oxidation occurs between 4% and 55 °C (Stevenson 1986), a range well within that normally observed in soil systems. Therefore, the sulfur oxidation rate is generally directly proportional to the temperature in soil systems (Ahonen and Tuovinen 1990; Attoe and Olson 1966; Chapman 1989; Janzen and Bettany 1987c; Nor and Tabatabai 1977). A wide range of Q_{10} values was reported in these studies: 1.9–3.1 (Chapman 1989), 2.1 (Ahonen and Touvinen 1990), 3.2–4.3 (Janzen and Bettany 1987c).

15.4 Biological Sulfur Reduction

As is the situation with reduction of nitrate in the nitrogen cycle, sulfate can be reduced via both assimilatory and dissimilatory pathways. Assimilatory sulfate reduction is the source of sulfur for biomass synthesis. Therefore, this sulfate

reduction process is catalyzed by a variety of organisms, including higher plants, algae, fungi, and bacteria. In contrast, dissimilatory sulfate reduction, sulfidogenesis, is catalyzed by a specialized group of obligately anaerobic bacteria. In soil, the organisms involved with the latter reaction are generally found in the genera *Desulfovibrio* and *Desulfotomaculum*. As with nitrate in denitrification, sulfate is the terminal electron acceptor for dissimilatory sulfate reducers. In both dissimilatory processes, the electrons are transferred through a cytochrome-based electron transport process to the terminal electron acceptor.

With dissimilatory sulfate reduction, sulfate is reduced through the transfer of eight electrons produced by the oxidation of a fixed carbon substrate, as described by the following reaction:

$$SO_4^{2-} + 8e^- + 8H^+ \rightarrow H_2S + 2H_2O + 2OH^-$$

The sulfate atom is activated in this process through the consumption of adenosine triphosphate (ATP). A variety of fixed carbon compounds may be oxidized to provide the electrons for this process. The most commonly studied energy sources are organic acids (e.g. lactate, acetate, fatty acids), but an increasing number of more complex substances, such as various aromatic compounds, are being added to the list of electron donors. Coupling of this process with mineralization of organic soil pollutants is being explored as a means of bioremediation of soils.

Dissimilatory sulfate reduction in soil systems is distinguished by its occurrence only in specialized ecosystems. Aside from the requirement for an oxidizable substrate and sulfate, the primary environmental property delimiting function of these anaerobic bacteria is anoxic conditions. Sulfidogenesis is only detected in soil ecosystems exhibiting extremely reducing conditions. A reducing potential of about −0.25 V is required before dissimilatory sulfate reduction occurs. Thus, this sulfate reduction process is limited to sites similar to those where methanogenesis occurs, i.e. swampy or chronically flooded soils.

Although sulfate is a common component of soil interstitial water, its presence in concentrations supportive of extensive dissimilatory sulfate reduction is limited. A typical example of a land-based ecosystem that would allow extensive sulfide generation is a marsh site inundated with sea or brackish water. Anoxic conditions prevail in the marsh soils and sulfate is a major anion in seawater. The best indication of current or previously existent sulfidogenic conditions is detection of metal sulfide deposits in the soil profile or detection of hydrogen sulfide fluxes from the site.

It should be noted that although the primary substrate considered for sulfidogenesis is sulfate, elemental sulfur can also be reduced by a variety of microorganisms. For example, *Desulfurolobus ambivalens* has the capacity to oxidize or to reduce elemental sulfur (Zillig et al. 1986). *Desulfotomaculum termoacetoxidans* (Min and Zinder 1990), *Desulfuromonas acetoxidans* (Gebhardt et al. 1985), and *Spirillum* 5175 N (Zoephel et al. 1988) all reduce elemental sulfur to hydrogen sulfide.

Sulfidogenesis may be managed to ameliorate the impact of a variety of environmental pollutants, both organic and inorganic. For example, this process may be explored as a means of reducing organic contamination of chronically flooded soils, reducing drainage water acidity, and ameliorating the difficulties associated with heavy metal contamination of soils and waters.

15.4.1 Anaerobic Biodegradation

A variety of xenobiotic compounds containing halogenated aromatic rings may enter soil ecosystems in undesirable concentrations. Under aerobic conditions, the halogenated substances are reasonably stable but under anoxic conditions, bacteria have been shown to be capable of removing the halogen atom. Since at least some of these substances are mineralized by the sulfidogenic bacteria, the combination of dehalogenating and sulfate-reducing populations may be used to remove this troublesome class of compounds from sites that can be easily managed under anoxic conditions. These techniques may be particularly operative in sediments polluted with xenobiotic compounds.

15.4.2 Reducing Acidity of Acid Mine Drainage

Acid mine drainage results from exposure of previously buried sulfide-bearing soil minerals to oxidative conditions in the presence of an active sulfur-oxidizing population. The environmental impact of the highly acidified waters is further accentuated by their high metal loadings resulting from the dissolution of the soil and mine slag minerals. Thus, soils and waters receiving these drainage waters are adversely impacted not only by the acidic conditions but also by toxic levels of heavy metals. A means of reducing the environmental impact in these situations is to pass the drainage waters through a swampy area where dissimilatory sulfate reducers can convert the sulfate to sulfide. The metal concentrations of the waters are reduced by their precipitation as metal sulfides. See Mills (1985) for a more detailed discussion of this process.

15.4.3 Reduction of Complications of Metal Contamination in Soil

Sulfidogenesis can also be exploited in situations where soils have been contaminated by heavy metals. As with the acid mine drainage problem outlined above, metal loadings of the leachate waters from the contaminated site may be removed by precipitation as metal sulfides in a sulfate-reducing system. Mobility of the metals may also be reduced in the site through creation of appropriate conditions for sulfide genesis within the soil profile. The primary difficulty with both the utilization of sulfidogenesis to remove metal contaminants from acid mine

drainage and to reclaim metal contaminated sites is that the anoxic conditions of the purification site must be maintained. Should the soil become aerobic, sulfur-oxidizing bacteria will utilize the sulfide for an energy source, thereby mobilizing the previously stabilized heavy metals.

15.5 Mineralization and Assimilation of Sulfurous Substances

Mineralization of organic sulfur compounds is a microbially catalyzed process analogous to carbon and nitrogen mineralization. Although the relative rates of nitrogen, carbon, and sulfur mineralization in a specific soil system may differ (see Bettany et al. 1980 for an example of a field study and Tabatabai and Al-Khafaji 1980 for a laboratory-based study demonstrating this difference), each is dependent upon activity of a variety of heterotrophic bacteria and fungi. This commonalty of mediators results in a similar impact of variation of environmental conditions on process rates. That is, sulfur mineralization is anticipated to occur in any ecosystem wherein heterotrophic microbes can function. Under aerobic conditions, complete mineralization of an organic substrate yields carbon dioxide, ammonium, and sulfate. Under anoxic conditions carbon dioxide, methane, sulfide, and ammonium are primary products. Optimal conditions for mineralization of sulfur-containing organic compounds include a near neutral pH, mesophilic temperatures, and approximately field capacity soil moisture. It is easily concluded *a priori* that since sulfur mineralization occurs over essentially the totality of the physical and chemical conditions supportive of microbial life, the process is catalyzed by an extremely diverse group of common soil bacteria and fungal species.

The mineral sulfur produced through the catalytic activity of the soil decomposer community may be assimilated by the plant community or immobilized (assimilated) by the soil microbes. Since ecosystem conditions generally favor assimilation of sulfate into plant or microbial biomass, the bulk of the sulfur contained in soil systems tends to be in the organic fractions (e.g. David et al. 1982; Swank et al. 1984). Since these assimilatory processes are catalyzed by the plant community and the heterotrophic soil bacteria and fungi, the physical and chemical limitations to the activities would be similar to those previously described for overall soil microbial activity.

As has been observed with carbon and nitrogen mineralization, the rate of mineralization of sulfur compounds is proportional to the biodegradation susceptibility of the organic compound itself as well as its physical availability to the degrader population. That is, the decomposition rate is directly dependent upon the capability of the microbial population to synthesize the requisite enzymes necessary for the catalysis and the proximity of the organic substance to the active microbial

population. Since both biodegradation-sensitive (common cellular substituents) and more biodegradation-resistant sulfurous compounds (humified substances) are found in soil (e.g. Anderson et al. 1981 or Bettany et al. 1979), the conversion of organic sulfur to sulfate in an aerobic soil system may or may not be proportional to the total soil organic matter content. This relationship is totally dependent upon the relative contribution of biodegradable and accessible organic sulfur to biologically stabilized (e.g. humic acid) sulfur.

15.6 The Phosphorus Cycle

Processes associated with transformation of phosphorus occurring in soil occur by the same principles as the other soil-based biogeochemical cycles. Mineralization and assimilation are catalyzed by the general soil biological community. The phosphorus cycle, like the sulfur cycle, involves the interactions of mineral and organic nutrient forms, with the quantity of water-soluble phosphate being controlled not only by biological mineralization processes but also by dissolution rates from soil mineral fractions. Unique aspects of the phosphorus cycle include the fact that phosphorus does not undergo any valence changes in the cycle and that there is no gaseous component to the cycle. Based on the similarities of the biological and chemical properties of the phosphorus cycle to those of other nutrient cycles, only the salient features of the phosphorus cycle will be described herein. For a more detailed analysis of the nuances of the phosphorus cycle, see Stevenson (1986).

In a depiction of the phosphorus cycle with biomass accentuated, the pivotal process is mineralization of organic matter into plant and microbially available phosphate (Figure 15.3). As with the mineralization processes central to the carbon, nitrogen, and sulfur cycles, phosphorus mineralization is catalyzed by the general soil heterotrophic microbial population (e.g. Molla et al. 1984). Thus, as in the previously described process, the mineralization rate is controlled by the physical and chemical factors delimiting overall microbial heterotrophic activity. Phosphate mineralization and its assimilation into biomass are generally optimal under those soil conditions suitable for aboveground plant community development. Mineralization occurs under both aerobic and anaerobic conditions, but maximal activity is detected in the presence of molecular oxygen.

A variety of relatively easily decomposed organic phosphorus compounds are found in biomass. The most abundant phosphatic substances are inositol phosphates, which make up 10–50% of the organic phosphate fraction. Other organic phosphates are phospholipids (1–5%) and nucleic acids (0.2–2.5%) (Stevenson 1986). Lesser concentrations of phosphopyridines (e.g. NAD and NADH) and nucleotides (e.g. ATP) are detectable in soil organic phosphorus fractions.

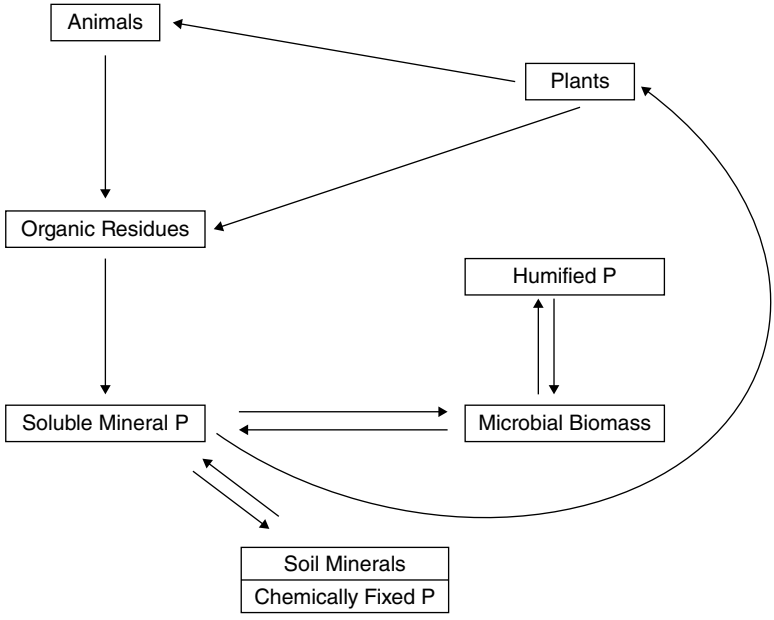

Figure 15.3 Major soil phosphorus cycle reservoirs.

Native phosphate in soils is derived from rock phosphate, apatite, which has the empirical formula $3(Ca_3(PO_4)_2)\cdot CaX_2$ where X equals Cl^-, F^-, OH^- or CO_3^{2-}. Secondary minerals that are formed from phosphate in soil are wavellite ($Al_3(PO_4)_2(OH)_3\cdot 5H_2O$), variscite ($Al(PO_4)\cdot 2H_2O$), dufrenite ($FePO_4\cdot Fe(OH)_3$), strengite ($Fe(PO_4)\cdot H_2O$), and vivianite ($Fe_3(PO_4)_2\cdot 8H_2O$). Along with the effect of phosphate mineralization rate, the quantities of plant-available phosphate present are determined by the solubilization rate of phosphatic minerals. Organophosphate levels in soil range from 15% to 80% of the total phosphates (Stevenson 1986). Therefore, in soils with high soil organic matter pools, the phosphorus needs of the plant community can be readily met via turnover of living biomass and mineralization of available soil organic matter pools whereas in the contrasting situation, with high mineral phosphate and low organic matter levels, the rate of solubilization of the minerals would be anticipated to be a major controller of plant growth.

Soil microbes are not only mediators of phosphate biological mineralization and immobilization processes, but they can also be instrumental in solubilization of rock phosphates via the synthesis and release of organic acids and carbon dioxide into the soil system. The organic acids liberate phosphate through chelation of the associated metal ions in the phosphatic minerals. Carbon dioxide contributes to the acidity of soil water by formation of carbonic acid. As the

pH of an acidic soil increases, organic acid production becomes increasingly important in determining dissolution rate of rock phosphates (e.g. Jayashree et al. 2011; Traina et al. 1986).

Were this the end of the story, understanding the ecosystem processes affecting phosphate transformations in the soil environment would be easy. Differential levels of water-soluble and insoluble phosphate arise from the fact that a variety of ecosystem properties control soil phosphate distribution within and between soil phosphate pools. Examples of additional ecosystem properties affecting phosphate availability include soil water contents (drought or wet conditions) (Dijkstra et al. 2015), the nature of the plant community (including intercropping) and the relationship of "hot spots" of phosphatase activity in the vicinity of the rhizosphere (Bettencourt et al. 2012; Spohn et al. 2015; Tang et al. 2014). Increased solubilization of phosphate minerals has also been proposed to result from inoculation of the soil with organic acid-synthesizing microbes (Giles et al. 2014).

15.7 Microbially Catalyzed Soil Metal Cycling

Soil-resident reservoirs of biomass-nutrient cations (as well as toxic cations) are easily grouped into three categories: contained in biomass, dissolved in interstitial water, and retained in soil minerals (i.e. water-insoluble forms) (Figure 15.4). Biomass productivity may be controlled by existence of excessive quantities (toxic metals) and occurrence of minimal levels (required nutrients). The soil biological community is instrumental in catalyzing a variety of processes that directly or indirectly result in the transfer of cations between these three soil reservoirs.

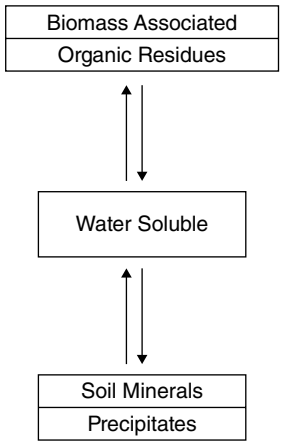

Figure 15.4 Primary metal reservoirs in soil.

15.7.1 Interactions of Soil Metals with Living Systems

Benefits from maintenance of appropriate levels of cations in interstitial waters result from their essential roles in cellular biochemistry. Plant essential trace metals include iron, zinc, manganese, calcium, boron, molybdenum, and, most likely, nickel. Animals also require cobalt, chromium, and tin. In contrast, cations of major interest in relation to negative interactions with biomass production include cadmium, mercury, and lead.

In most ecosystems, the balance between aboveground productivity and trace metal availability is maintained by the movement of soil-resident cations between the various pools (Figure 15.4). Limitations of these plant nutrients to overall biomass production are generally restricted to ecosystems where augmentation of biomass production above that essentially dictated by native soil mineral cycling rates is necessary (i.e. intensive agricultural production) or in cases where metals are removed from the system through harvesting of aboveground biomass. In these situations, aboveground biomass needs are met through external amendments and/or modification of soil pH to optimize availability of soil resources.

In contrast to the situation exemplified by intensive agricultural systems, metal limitations for microbial productivity in soil are generally a rarity. Soil microbes possess highly efficient mechanisms for transport of cations into the cell. Furthermore, as discussed below, many members of the soil microbial community can produce chelators that increase water-soluble cation resources.

An increasing array of environmental problems are arising where management of the soil for stimulation of ecosystem sustenance and/or development results in alteration of metal availability to the microbial community. Historically, the most commonly cited toxicity problem involving soil cations is associated with aluminum concentrations in acidic soils. Aluminum is toxic to biological processes. As the soil pH declines, the solubility of aluminum increases. Thus, best management practices of acidic soils for crop production have frequently included addition of lime to the soil to raise the pH as well as amendment with organic matter to complex the water-soluble ions (see Stevenson 1982 for a more complete analysis of aluminum solubility in soil). More recent considerations involving toxic metals in soil have been associated with environmental contamination (e.g. smelter and foundry operations) and from disposal of societal wastes (e.g. land disposal of metal-contaminated biosolids and composts).

The implications of increased soil loadings of toxic cations on ecosystem function are best exemplified by the succession of aboveground and belowground communities associated with metal contamination of soils by smelter operations. The availability of the toxic cation may become the controlling factor in community development, as was observed by Nordgren et al. (1983) in soils near a brass mill in South Sweden. In these soils sampled along a copper and zinc gradient

(with maximum levels of 20 000 μg copper and zinc g^{-1} soil), the influence of soil organic matter content and moisture on fungal community composition was minimal compared to the effect of heavy metal pollution on these populations.

A more general impact on ecosystem communities has been noted for heavy metal-contaminated soils near a zinc smelter at Palmerton, Pennsylvania. Soil heavy metal loadings (litter horizon within 1 km of the smelter) in the 1970s included about 26 000 μg zinc g^{-1} soil, 10 000 μg iron g^{-1} soil, 2300 μg cadmium g^{-1} soil, and 340 μg copper g^{-1} soil (Strojan 1978) with as much as 90% of the metals being retained within the top 15 cm of the soil profile (Baucher 1973). These heavy metal loadings declined to approximately one-tenth these values at a control area about 40 km east of the smelter. In this 1978 study, consideration of the average amount of organic matter in the more highly polluted site and comparison of decomposition rates of leaf litter in litter bags led to the conclusion that organic matter decomposition rates were reduced by the heavy metal contamination. In a 1975 report (Jordan 1975), reduced germination of tree seeds due to high zinc concentrations was proposed as a mechanism preventing the establishment of invader tree species in regions where indigenous trees, shrubs, and herb populations were in decline from the metal contamination. Additionally, in this site, the prevalent metal contaminant concentrations exceed those necessary to result in major reductions of total bacterial, actinomycete, and fungal populations (Jordan and Lechevalier 1975). Major alterations of the soil microbial as well as aboveground populations resulted from the metal contamination as demonstrated by changes in the soil metabolic diversity (Kelly and Tate 1998) and phospholipid fatty acid profiles (Kelly et al. 2003).

Currently, the aboveground status of the site at Palmerton can be defined as an "establishing" grass community due to overlaying of the soil with a sludge-fly ash-limestone-grass seed mixture or a essentially barren system in untreated sites (Figure 15.5). The once flourishing forest ecosystem has been reduced to a bare mountainside. Reclamation of the site requires redevelopment of a functional soil microbial community accompanied with establishment of aboveground plant community consisting of a grass community.

With both of these examples, extremely high metal loadings were detected in the environmental samples. It must be remembered that the toxicity of the metals is proportional to their availability to the microbial community. Thus, inhibitory concentrations of metals derived from pure culture study where all the toxicant is water soluble frequently do not correspond to field observations where total soil metal concentration can be distributed between a variety of chemical forms that vary in availability to the soil microbial community (e.g. Lighthart et al. 1983). In a related study (Sullivan et al. 2013), variations in soil chemical properties and soil bacterial and archaeal community composition were strongly related in a muck soil with elevated sulfur and metal content. Therefore, when evaluating metal

Figure 15.5 Metal-contaminated site in Palmerton, PA (USA), showing effect of organic matter, lime, and fly ash amendments on grass growth.

loading toxicity, soil properties that alter metal distribution between water-soluble and water-insoluble species must be considered. For example, in a study of planktonic, sediment and epilithic bacterial community adjustment to heavy metal concentrations (Dean-Ross and Mills 1989), changes in microbial populations did not reflect the known toxicity of the contaminating metals. It was concluded that the occurrence of high pH in the samples that reduced the solubility of the toxic metals apparently resulted in lack of correlation between metal concentrations and response of the bacterial communities to the toxicants.

The meaningful impact on microbial community structure in soils containing high loadings of toxic metals is reasonably anticipated *a priori*, but long-term effects on microbial community structure in soils receiving moderate to low levels of such metals have also been reported. For example, MacDonald et al. (2011) found that changes in bacterial and fungal community structure resulting from zinc- and copper-containing sewage biosolides were detectable more than 11 years post amendment,

15.7.2 Microbial Response to Elevated Metal Loading

The most obvious effect of high metal loadings on the soil microbial community is toxicity. Death of sensitive microbes and plants may result directly from dissolved cations as well as indirectly from the release of cations following catabolism of the

organic matter to which they are bound. As implied by these data, alteration of soil microbial community dynamics and functions can be anticipated to result from soil contamination with heavy metals. A primary impact of metal contamination is the inhibition of catabolism of organic substances by the toxic metals.

Microbial populations may be inhibited by release of organic matter-associated cations through microbial catabolism of the organic matter. The microbial recovery of energy from the fixed carbon substances results in accumulation of toxic concentrations of the metal. This phenomenon is exemplified by studies of the decomposition of iron, aluminum, zinc, and copper salts complexed to polysaccharides (Martin et al. 1966), metal-nitrilotriacetate (calcium, manganese, magnesium, copper, zinc, cadmium, iron, and sodium chelates were examined) complexes (Firestone and Tiedje 1975) as well as copper, mercury, zinc, and cadmium salts of 2,4-dichloro-phenoxyacetic acid methyl ester (Said and Lewis 1991). As the concentration of the metal liberated from the organic complex increased, microbial activity decreased. The degree of inhibition varied with toxicity of the metal to the decomposer populations.

Overall reduction of microbial respiration by increasing concentrations of a variety of heavy metals is exemplified by the study of Lighthart et al. (1983). Additionally, examples of the capacity of heavy metal contamination to reduce or inhibit soil biological processes include a variety of enzymatic activities (e.g. Cole 1977; Doelman and Haanstra 1979, 1989; Stott et al. 1985) as well as various soil nitrogen transformations (immobilization, mineralization and nitrification (Chang and Broadbent 1982), anaerobic nitrogen transformations (Blais et al. 1988), and nitrogen fixation (Wickliff et al. 1980).

A variety of organisms must exist in soil with augmented resistance to heavy metal pollutants. For example, soil bacteria have been isolated with augmented resistance to metal contaminants, including cadmium (Bopp et al. 1983), copper (Dressler et al. 1991; Yang et al. 1993), mercury (Kelley and Reanney 1984), nickel (Schmidt et al. 1991), and silver (Haefeli et al. 1984). Selective enrichment of metal-tolerant bacterial populations in polluted soils has been demonstrated with classical microbiological procedures (e.g. Duxbury and Bicknell 1983) as well as DNA probe-mediated methods (e.g. Diels and Mergeay 1990).

15.7.3 Microbial Modifications of Metal Mobility in Soils

Soil properties controlling the availability of both essential nutrient cations and toxic metals are primarily soil pH, redox potential and organic matter concentrations. Soil-buffering capacity, chlorinity, inorganic cations and anions, clay mineralogy, and moisture (e.g. Babich and Stotzky 1983) also contribute to metal solubility. The microbial community is instrumental in modifying each of these

soil factors in a manner that can enhance or reduce soil interstitial water metal loadings. Furthermore, microbial interactions affecting metal mobility in soil can be grouped as indirect or direct. Indirect interactions include pH alteration, siderophore production, and modification of the physical environment (e.g. changing the redox potential). Direct interactions include valance change, substitution, methylation, transalkylation, incorporation into cell substituents, and release from organic association through mineralization.

15.7.3.1 Acidification and Chelation

Soil microbes produce a variety of inorganic and organic acids that contribute to solubilization of metals. The inorganic acids could be considered to be by-products of energy metabolism. Two of the more commonly encountered products are carbonic and sulfuric acid. Aerobic microbes yield carbon dioxide as their terminal oxidation product. This gaseous substance exists in equilibrium in soil between carbonic acid, bicarbonate, and carbonate. Carbonic acid is a weak acid capable of dissolving soil minerals.

A potentially more dramatic example of mineral acid production is the sulfuric acid produced by oxidation of sulfides and elemental sulfur by soil thiobacilli. This process has been exploited by recovery of mineral from ores (e.g. Carlson et al. 1992) and has been proposed as a means of removing mineral sulfides from municipal sewage biosolids (e.g. Jain and Tyagi 1992). An example of ecosystem destruction resulting from sulfur oxidation by soil microbes and alteration of metal solubility production is the orange-colored leachate associated with acid mine drainage (e.g. Mills 1985).

Organic acids that may solubilize soil minerals either directly through acidification of the microsite or through chelation reactions are produced by aerobic and anaerobic soil microbial processes. Under anaerobic conditions, a variety of organic acids accumulate due to incomplete oxidation of fixed carbon substrates. Commonly produced substances under aerobic conditions are siderophores. These chelators are important in iron nutrition of bacteria and higher plants. Bacterially synthesized siderophores may be instrumental in solubilizing significant quantities of iron for higher plant biomass synthesis. Such substances produced by rhizosphere bacteria have been shown to stimulate associated plant productivity (e.g. Kloepper et al. 1980). Similarly, rhizobial siderophores have been shown to stimulate clover productivity (Derylo and Skorupska 1992). A large number of members of the general soil community also produce these metal chelators, for example *Pseudomonas* species (e.g. Ankenbauer et al. 1988; Bar-Ness et al. 1991; Cody and Gross 1987; Hoefte et al. 1991). The fact that these substances are synthesized within the soil ecosystem is supported by the capacity to isolate a variety of such substances from soil samples (e.g. Akers 1983a, b; Powell et al. 1982).

15.7.3.2 Direct and Indirect Oxidation and Chemical Reduction of Soil Minerals

Oxidation of metals by soil bacteria is exemplified by the oxidation of ferrous ion by *T. ferrooxidans* and mercury oxidation by a variety of bacteria (e.g. Holm and Cox 1975) as well as by the oxidation of metal oxides (e.g. conversion of arsenite to arsenate by *Alcaligenes faecalis* [Phillips and Taylor 1976]). Similarly, a number of bacterial species are capable of chemically reducing a number of metallic cations and metallic oxides, including iron (Lovley and Phillips 1986), manganese and iron (Nealson and Myers 1992), uranium (Lovley et al. 1991; Lovley and Phillips 1992), and chromate (Eary and Rai 1991).

Indirect modification of metal mobility by changing its valence state can result from microbial modification of the redox potential of the soil microsite. This process is envisioned in soil ecosystems where microbial energy metabolism results in exhaustion of molecular oxygen, nitrate and other electron acceptors, creating the highly reducing condition necessary for spontaneous chemical reduction of metals (such as ferric ion reduction to ferrous iron). The result of these reactions frequently results in changes in soil physical structure. For example, iron reduction in waterlogged soils can result in gley formation (see Tate 1987). In this situation, the precipitation of the ferrous ion by the concurrently formed sulfide causes a gray-to-black coloration of the soil. This alteration of the soil color within the profile can typically be used to determine transient occurrence of a high water table and the resulting imposition of anoxic conditions.

An interesting indirect modification of oxidation state of metal compounds may occur in the rhizosphere of plants that actively oxygenate their root zone. These plants, such as rice, transport molecular oxygen from the aboveground tissue into the root zone. Oxidation of technetium in rice (*Oryza sativa* L.) was shown to result from this mechanism (Sheppard and Evenden 1991).

15.7.3.3 Bioaccumulation and Solubilization of Metal Ions through Mineralization

As is true of all living cells, soil microbial biomass incorporates essential metals into cell substituents. Also, accumulation of metallic cations on or within living cells can greatly exceed these metabolic requirements. This bioaccumulation is delineated by the achievement of cellular loadings of metals far beyond any levels that might be associated with cellular metabolism. This process is exemplified among the bacterial populations by strontium accumulation by *Micrococcus luteus* (Faison et al. 1990) and precipitation of cadmium by *Clostridium thermoaceticum*. In the latter example, cadmium is accumulated at the cell surface as well as in the surrounding medium (Cunningham and Lundie 1993). Similarly, cesium (Tomioka et al. 1992) and zinc (Sakurai et al. 1990) can be accumulated by bacteria. It must be noted that the cells need not be living for augmentation of cell-associated metals

to occur. Kurek et al. (1982) found that dead microorganisms were capable of absorbing more cadmium than did the live cells used in their studies.

The return of biomass-associated metals to soil water-soluble or mineral forms occurs through mineralization of the microbial biomass following death of the cell. As opposed to the cycling of carbon, nitrogen, and sulfur discussed above, the return of the metal to a mineral state likely provides little benefit to the decomposer population and is generally not directly enzymatically mediated. This metal cycling could be classified as a gratuitous product of microbial cycling of carbon.

15.7.3.4 Other Soil Metal Transformations

As was indicated above, soil minerals may also be methylated and transalkylated by soil microbial populations. The attachment of alkyl groups to the metal may alter the metal mobility within the ecosystem through enhanced volatility and water solubility. For example, monomethyl and dimethylmercury are volatile. Therefore, their formation in soils can result in reduction in the level of mercury contamination a the specific soil site (see Tate 1987 for further discussion of the behavior of metals in soil ecosystems).

15.7.4 Managing Soils Contaminated with Toxic Metals

Metal-contaminated soils present a unique challenge to the soil microbiologist charged with the development of soil renovation plans. Conceptually, most microbial reclamation procedures involve management of the availability of the contaminant to the soil community of degraders and encouragement of mineralization of the offending substance. In contrast, reduction of elevated metal loadings involves either removal of the contaminant from the site (leaching or excavation of the soil itself) or modification of the properties of the polluted soil in a manner that encourages sequestering of the toxic metal into biologically unavailable forms. Since the three major soil properties controlling metal mobility are pH, redox potential, and organic matter content, traditional soil reclamation involving metal management has included manipulation of these parameters.

Some shifting of soil metals from water-soluble to particulate fractions can be achieved through soil amendment with lime and/or organic matter. Increase in soil pH to approximate neutrality through lime amendment encourages a change in equilibrium between soluble and insoluble metal forms. With the addition of organic matter, the soluble metal loadings are reduced through complexing of the metal with the organic material itself. (See Tate 1987 for a more detailed discussion of control of soil metals through these mechanisms.) These nonbiological management procedures only provide a temporary solution to the metal toxicity. For example, once the organic matter retaining the metal is decomposed, any associated cations are released into the interstitial soil water. Similarly, from the

view of management of soil pH, the natural tendency of soil systems is to become increasingly acidic. Thus, continued monitoring and adjustment of soil acidity may be necessary in particularly sensitive ecosystems if on-site retention of the metal contaminant is essential.

Neither of these procedures, pH adjustment and organic matter amendment, presents any difficulty to the development or continued function of the soil microbial community. Organic matter encourages the stability of soil decomposer communities through the provision of carbon and energy. Furthermore, site management may require raising the soil pH to approximating neutrality (i.e. within the range of optimal function of the majority of the members of the soil biological community), and negative effects on biological parameters are not anticipated.

A more intensive stewardship of metal-contaminated sites may actually involve alteration of the biological properties of the system. Activity of both the aerobic and the anaerobic microbial populations can be manipulated to encourage sequestering of metal contaminants. A logical extension of an understanding of the dynamics of sulfidogenesis and chemical interactions of the sulfide produced with soil metals is management of the cycle to purify soil interstitial waters. Provision of reducing conditions in the presence of sulfate ions may allow production of sufficient sulfide to reduce soluble metal loadings to acceptable levels. Metal management through control of the sulfur cycle is commonly accomplished by creation of artificial wetland. The difficulty with this procedure is that the wetland created must be maintained in an anoxic condition. If oxygen is introduced into the anoxic wetland, sulfur oxidizers utilize the sulfide as an energy source, thereby liberating the associated metal ion.

15.8 Conclusion

The foregoing analysis of the sulfur, phosphorus, and mineral cycles in soils underscores not only their essentiality to total ecosystem biomass productivity but also a primary role in reversal of adverse situations resulting from anthropogenic activities. That is, excessively acidic soils may result from elevated influxes of reduced sulfur compounds, heavy metal contamination may preclude sustenance of soil microbial communities, and mobility of soil phosphate may accelerate eutrophication of regional surface waters. Ameliorating these unfavorable soil or water conditions, while optimizing biomass productivity in soil ecosystems containing restrictive concentrations of the essential components of these cycles, requires a firm appreciation for not only the biological aspects of the biogeochemical cycles but also the chemistry of formation and dissolution of soil minerals.

These biogeochemical cycles are highlighted by the major role of soil primary and secondary minerals in maintaining concentrations of bioavailable water-soluble nutrients. This intersection of the mineral with the biological world and the implications of societal mismanagement of soil ecosystems involving soil phosphate, sulfur, and metal loadings can be managed to encourage development of sustainable ecosystems.

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16

Soil Microbes

Optimizers of Soil System Sustainability and Reparation of Damaged Soils

Soil management, as well as the changes that naturally impact soil function with evolution of a soil-based ecosystems, also affect systems associated with atmospheric process down to the more limited terrain of a backyard garden. An excellent example of this keystone relationship is exemplified by the large variety of microbes and higher plants affecting the size of soil aggregates and the rate of their formation. As introduced in Chapter 1, soil aggregation is a process that affects a wide variety of soil properties, such as, influx and efflux of soil gases, including those associated with the atmospheric processes leading to climate change (e.g., carbon dioxide, methane, nitrous oxide, and nitrogen oxides.). Burke et al. (2012) provide an example of how the variety of microbial functional groups can impact the fate of greenhouse gases in soil. Additionally, examples of the complexity and diversity of these interactions between plant community and soil minerals and management are discussed by Poirier et al. (2018). Indeed, soil microbes are major contributors to ecosystem function in general. As stated by Comerford et al. (2013)

> "At this point of our examination of occurrence and functions of microbes in soil, one could easily reach the conclusion that the primary role of microbes in soils involves optimization of food and fiber yields, as revealed with both laboratory and field study. Such research has and will always contribute to our understanding of the importance of maintaining a healthy soil microbial community existence in a soil system but, in reality the soil microbes play a significant role in supporting all life on our planet. The all-encompassing importance of soil mineral-microbial interactions is a major support for all aspects of survival and sustainability of soil systems, be they for cropping, industrial usage, or are simply in jeopardy due to basic human societal activities."

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Thus, with these considerations of the role of microbes in bioremediation of all soils impacted by society in a negative manner, this chapter is presented as a capstone analysis of the importance of management of the microbial community for maximizing the productive and sustainability of soil systems in general and damaged soil systems specifically. It would perhaps be useful to consider the question "What are the types of systems that should be considered in our analysis of the types of human impacted ecosystems that should be considered in this analysis? Major human societal impacts soil organic matter can easily be traced to the times of the agricultural revolution (See Chapter 1). Many examples of situations where the levels of soil organic matter were reduced sufficiently to impede crop production—even a new, different type of ecosystem. Today, it is recognized that agricultural development and the subsequent loss of soil organic matter has resulted in the realization that cropping of soils is a major source of atmospheric carbon dioxide, therefor making soil a significant player in climate change. Optimization of best management plans for soils is a critical factor in managing of atmospheric carbon dioxide levels.

A soil ecosystem requiring bioremediation can be conceptualized in part as one in which the existing biological community has failed to function as might be anticipated. In a non-disturbed system, biologically decomposable substances entering the soil are mineralized. The concentration of the residual organic material is the difference between the amendment and decomposition rates. In a chronic situation, it is reasonable to assume that some detectable equilibrium concentration of the soil organic amendment will be achieved. If the input ceases or the material is added at long time intervals, it is equally reasonable to anticipate that the added substance could be totally eliminated periodically. In contrast, in our hypothetically polluted soil, a toxic substance has accumulated to concentrations that inhibit and/or interfere with the normal function of the ecosystem; that is, the system failed. Note that this statement does not exempt anthropogenic management failure from consideration. People are part of and major influences on the ecosystem. Thus, the failure of the microbial community to rid its environment of a toxic organic material could be a result of its being overwhelmed by mismanagement of the soil.

Considering that the pollutant accumulated to toxic levels, we can conclude that normal soil processes are inadequate to ameliorate the situation. The inability of the soil microbes to prevent the accumulation of the organic pollutant could be due to a number of factors:

- No individual or groups of microbial species exist in the soil capable of mineralizing or detoxifying the contaminant.
- The rate of input of the pollutant may be greater than its decomposition rate, resulting in ecosystem degradation.

- Chemical, physical, or biological limitations of the decomposers may exist or may be created by the contamination of the soil system thereby preventing microbial removal of the pollutant (e.g., anoxic conditions may develop preventing occurrence of obligatory aerobic processes).
- The pollutant(s) were amended to the soil at levels that are toxic to the decomposers themselves.
- Conditions may be optimal for decomposition to occur, but the decomposer population could be physically separated from the decomposable pollutant. That is, the organic compound may be physically or chemically sequestered.
- Decomposition of the pollutant may create conditions in the soil wherein further mineralization is precluded (e.g., acidification of the system, depletion of molecular oxygen).

The objective of bioremediation is to manage soil physical, chemical, and biological properties in a manner that overcomes these limitations to biological activity and optimizes the capability of the microbial community to detoxify the soil.

The scientific basis for the management of the soil microbial community for remediation is built on the same biological and chemical foundations as occur in non-polluted soil systems. This commonality between functional native systems and those with excessive accumulations of toxic organic chemicals is exemplified by considering the processes involved with organic matter accumulation in soil. Amendment of any soil with plant biomass fuels the activity of the biological community and results in the production of microbial biomass and humus. With a normally functioning soil system, this accumulation of organic matter has a positive influence on ecosystem stability. (Although of course, there is a limit to the amount of organic matter that can be accumulated in soil.) Incorporation of plant biomass components into soil humus results, in part, from covalent linkages with soil humic substances. The organic components may also be occluded from decomposers by diffusing into soil micropores, by non-covalent stabilization within the three-dimensional structure of humic acids, or by sorption onto soil minerals. Since these are time dependent processes, a slowly mineralized substance has greater chance of becoming sequestered or sorbed onto or within clay or humic substances than do more biodegradation susceptible compounds. Thus, an easily metabolized organic compound, such as glucose or a protein, is normally fully mineralized by the soil microbial community in a short time, whereas cellulose and lignin of plant biomass are commonly stabilized within the soil structure. Also, small pieces of intact plant tissue can be protected from microbial attack by becoming encased in soil aggregates. In this situation, the sequestration results from physical adhesion of the soil particulate to small pieces of plant material. From the view of the normally functioning soil and non-toxic organic substances, these organic matter stabilizing processes result in enhancement of the soil system or an improved soil quality. In

contrast, similar protection of toxicants can result in ecosystem degradation and a reduction of ecosystem or environmental health. The processes are the same, but divergent consequences to the ecosystem function occur.

In the negative case, disruption of soil aggregate structure can actually become a positive management solution. Encouragement of accelerated decomposition of preexisting toxicants may result in not only lower pollutant levels but also in positive aspects of soil structure (such as, disruption of existing soil aggregation levels). In this situation, the availability of the pollutant molecules can be increased via liberation of substances previously protected within soil aggregates. In the latter situation, reduction in or destruction of the soil aggregate structure could be considered in the management plan initially to liberate the hidden pollutant followed by management of the soil to improve soil structure. The latter objective could be accelerated by introduction of plant communities. Note that this analysis primarily involves studies of remediation of surface soils.

Contamination of a soil ecosystem with biodegradable organic substances creates an unique situation for the microbes involved with their decomposition as well as for the site manager charged with evaluating the potential for bioremediation and the associated ecosystem risk. Thus, this chapter is presented with the overall objective of providing a foundation for considering the biological, chemical, and physical principles underlying or controlling the potential for bioremediation of soil ecosystem and the ultimate limitations or risks associated with such remediated systems. The soil amendment can range from simple organics (singly or in complex mixtures, biochar and biochar amended fertilizer, or plant nutrients in complex mixtures. (for examples of these materials used in the field, see Cartmill et al., 2014; Chen et al. 2014; Gandarillas et al., 2019; Lin et al. (2008); Zhou et al. 2019). This analysis will necessarily commence with a consideration of the microbes involved, but the primary emphasis will involve consideration of the physical and chemical limitations of the processes as they relate to bioavailability of the contaminant. Note that the topics analyzed herein tend toward analysis of basic concepts of microbial bioremediation. Basically, this chapter will extend upon the analyses presented in the proceeding chapters with a goal of providing a foundation for a more detailed examination of the bioremediation principles as presented specifically in the bioremediation literature.

16.1 Foundational Concepts of Bioremediation

16.1.1 Bioremediation Defined

Soil bioremediation is defined as the management or utilization of the soil biological community to detoxify, immobilize or mineralize organic contaminants in the soil ecosystem. Specifically, this process ideally involves conversion

of the pollutant totally to mineral forms (e.g., carbon dioxide, methane, ammonium, hydrogen sulfide, etc.), its conversion to forms with no significant negative health traits (generally viewed from a human health viewpoint, but this discussion includes the concept of "health" of the total biological community), and/or immobilization via covalent linkage to soil humic acids (humification). The biological community generally managed for bioremediation is the native soil microbial community, but higher plants (phytoremediation) may also be manipulated to enhance toxicant amelioration, especially for remediation of metal-contaminated soils. The primary emphasis of this chapter is management of soil microbes (including those of the rhizosphere) to optimize reduction of the risk associated with an organic toxicant in soil. These microbes may be indigenous to the contaminated soil system or amended to the soil during the remediation process. That is, the metabolic capabilities of the indigenous microbial community may be enhanced with genetically engineered (gems) or other laboratory derived or cultured microbial strains. Site management includes manipulation of soil chemical and physical properties to optimize both bioavailability of the pollutant and the microbial capacity to transform it. Frequently bioavailability is discussed in a limited sense—that is, in relationship to human health or risk. For this discussion, bioavailability will be considered the availability of the organic pollutant to the totality of the biological community, especially the soil microbes involved in its detoxification and/or mineralization.

16.1.2 Conceptual Unity of Bioremediation Science

The foundation for our understanding of the processes involved in bioremediation is derived from a unity of biochemical-microbiological-ecological principles. These supporting principles for bioremediation are as follows:

- *All relationships of comparative biochemistry apply equally in the axenic culture of microorganisms and in the field soil.* A compound mineralized by one or a combination of pathways or processes in culture can be anticipated to be similarly transformed in a field soil. A caveat to this rule is that the rate and extent of the transformation is controlled by the environmental situation of the microbes involved, including stresses imposed on the microbes through their interactions with other members of the biological community. Thus, the reactions may proceed at faster or slower rates and different intermediates may accumulate in the field due to limiting conditions (nutrient, physical, chemical or other). Only a small proportion of the soil microbes have been cultured in the laboratory and their biochemical capacities characterized. Variations in many biochemical processes that have yet to be described may exist.

- *All microbial growth requirements are the same whether in the laboratory culture and in the field.* If a growth factor is required in culture for a specific enzyme to function, it must also be supplied in the soil system. Similarly, the microbes and enzymes are controlled by the same physical limitations (e.g., pH, temperature) in the field and culture. The microbes may be functioning at the extremes of their growth margin—hence a slower process rate is seen—but, the organisms will not function beyond their normal range without some environmental compensation (a phenomenon also observed in axenic culture (See Chapter 5). For example, provision by root exudates or by other members of the microbial community of a cofactor whose *de novo* synthesis is precluded by high temperature may allow the microbe to function at a higher temperature.
- *All limitations resulting from ecological interactions apply, including the need to accommodate the presence of other microbes as well as adaptation to the physical and chemical properties of the microsite wherein the microbe lives.* This principle has most likely been the most overlooked when developing genetically modified strains or when selecting variant strains of microbes for soil inoculation. A microbe amended to a soil ecosystem with the intent of stimulation of bioremediation processes must not only possess the requisite genetic information, but be capable of expressing that capability *in situ*. Additionally it must also have the capacity to become, at least temporarily, a part of the overall soil microbial community. That is, the alien microbe must be able to express the requisite biochemical activity in the community, become established in that community, and accommodate or adjust to all means of interactions (ammensulism, competition, etc.) with its neighbors—at least until its presence is no long required for bioremediation.

These underlying principles of bioremediation processes underscore the fact that successful management of the soil microbial community to ameliorate contamination with organic pollutants is truly an interdisciplinary endeavor. All involved must have an appreciation of the basic concepts of soil science as well as the principles of soil microbiology, microbial physiology, and ecology. This interdisciplinary aspect of bioremediation technology is further accentuated when the impact of environmental engineers in applying the principles in the field is considered.

16.1.3 Complexity of Remediation Questions

Bioremediation involves mitigation of chemical contamination of soils and thereby a reduction of the impact of the presence of the pollutants on ecosystem function. By the nature of the vast array of organic substances and of the diversity in the extent of their encounter with soil systems, the soil system managed for

bioremediation can range from a few square meters to many hectares in area. The degree of intervention may range from the simple working of a compacted soil surface and amendment with fertilizer to the implementation of complex, engineered systems for pollutant retention and soil management developed to meet the needs for remediation of highly contaminated industrial sites. Definition of the breadth of chemicals contaminating soils extends far beyond the purview of this chapter, but the range in structural complexity can be gained from such reviews as that of Swobada-Colberg (1995) on xenobiotic chemicals.

Implications of the importance of bioremediation extend across not only those aspects associated with quality of the specific soil of interest, but also encompass more general concerns of environmental or ecosystem health. First concerns with bioremediation must involve mineralization of the organic substances of interest or immobilization of the inorganic substance, as is involved with phytoremediation of metal contaminated soils, but these initial endeavors must be executed with considerations of the effect of system management on the general health of the total environment in mind. That is, management of the decomposer population has implications far beyond inducing a simple reduction in soil toxicant loading. For example, utilization of mycorrhizal fungi as mineralizers of organic contaminants also alters nutrient and water dynamics of the associated plant community. This enhancement of the mycorrhizal association will result in a stimulation of plant biomass productivity, both aboveground and belowground. This increased plant growth will result in augmented activity of the rhizosphere community and an improved soil aggregate structure (see Chapter 1), primarily due to the stimulation of biological activity via exudate production.

A perhaps expected effect of the increased plant growth relates to soil water dynamics. In a soil system without a plant community, the direction of movement of the soil water, and any solutes (contaminants) contained therein, would be downward. With the increased water use of the plant in the system, a significant portion of the soil water is drawn to the root, thereby decreasing the quantity lost through percolation beyond the root zone. Thus, less pollutant is lost from the system by leaching to ground water and greater quantities are retained within the biologically activity zone of the soil (See Clothier and Green (1997) for a review of the impact of roots on soil water dynamics).

16.2 The Microbiology of Bioremediation

Consideration of the principles of bioremediation can be divided into two primary areas: a) Properties of the microbes themselves affecting their interaction with and decomposition of the pollutant and b) Properties of the ecosystem controlling the bioavailability of the toxicant. Successful biological remediation of

contaminated soils relies not only on the metabolic capability of the microbes but also on the probability of a favorable encounter between the toxicant and it decomposer community. That is, success depends on the bioavailability of the pollutant. Biodegradable organic substances entering a soil ecosystem can be mineralized at a reasonable rate (assuming no other limitation to microbial activity beyond those fulfilled by the organic amendment) as long as the pollutant is present within the soil interstitial water and existent in appropriate concentrations. Failure to fulfill the latter requirement can result in concentrations below that which allows efficient interactions with degradative enzymes as well as higher concentrations, which may be toxic to the microbe. Organic substances tend to have maximal concentrations in the available soil water immediately after entering the soil environment. During this time period, for metabolizable substances, the most probable fate of the organic substance is mineralization and incorporation of the carbon or metabolites into microbial biomass. A portion of the organic substance may be humified, sorbed onto soil minerals, leached through the profile, or volatilized. For bioremediation situations, this seemingly simple situation is made more complex by the fact that the distribution of the organic substrate becomes more complex with time, that is the proportion which becomes biologically unavailable increases with time in the soil system. Thus, for soil microbes, native or otherwise, to decompose soil contaminants, they must posses the genetic capability necessary to synthesize the requisite enzymes, be in an environment conducive to expression of their genetic potential, and have the capability to overcome the physical limitations of accessibility of the contaminant. The physical and chemical requirements for successful bioremediation of a soil site are the topic of the next section. This discussion will be limited to the properties of the microbial community that are requisite for successful biologically based remediation of chemically contaminated soil ecosystems.

16.2.1 Microbes as Soil Remediators

Soil microbes responsible for the decomposition of organic pollutants exist in three states:

- The decomposer populations are sufficiently high that immediate decomposition of the pollutant can occur. If the quantity of the pollutant soluble in the soil water is non-toxic and available at concentrations sufficient for effective catalysis, then degradation would be anticipated to occur—assuming that no other limitations (nutrients, oxygen, etc.) exit.
- Microbes capable of decomposing the contaminant are present in the soil system in low numbers. The pollutant may induce development of sufficient populations to allow the ultimate degradation of the pollutant. Major factors of

consideration in this situation include the time necessary for the population to develop (See Chapter 4 on microbial growth kinetics in soil) and the potential that other soil factors may limit the development of adequate microbial populations for biological remediation to occur.

- No indigenous organisms capable of decomposing the contaminant are present in the microbial community. In this situation, the potential to amend the soil with decomposer populations capable of surviving and functioning therein must be considered. This process may involve use of genetically modified organisms or simply adding soil or other amendment that contains the requisite populations. This process imposes an added limitation discussed above—the necessity of the alien microbes to overcome competitive interactions with the indigenous microbes. The existence of an "underutilized" carbon and energy source, the pollutant, in the system could provide an advantage to the amended or invading microbes.

If the appropriate organisms exist within the soil system for biological decomposition of the substance to occur, then there are several alternative processes that should be evaluated when assessing the potential for bioremediation. The microbes may metabolize the organic pollutant directly, with the product ultimately being mineralized through common intermediary metabolism pathways. The pollutant may be metabolized indirectly. In this situation, there may be no organisms present in the system capable of mineralizing the substance, but the contaminant may be transformed cometabolically and ultimately decomposed through the interaction of several members of the microbial community (a consortium). Further variation in the metabolic processes associated with transformation of the pollutant results from the microbial capability to utilize a variety of terminal electron acceptors. Thus, although aerobic decomposition of organic substances is generally initially considered in ecosystems contaminated with toxic substances, the capacity of bacteria to use alternative terminal electron acceptors, such as nitrate and sulfate, can be exploited to purify the soil. Each of these alternatives will be discussed in greater detail when the pathways for bioremediation are considered below.

16.2.2 Substrate-Decomposer Interactions

16.2.2.1 Physiological Pathways for Bioremediation

Description of specific pathways for biodegradation is beyond the scope of this chapter. (See chapter 4 for a general discussion of microbial metabolism in soil.) Examples of the complexity or variety of alternative pathways through which soil organic contaminants can be decomposed are provided by (Young and Cerniglia, 1995). Perhaps, the biggest mental obstacle to maintaining a clean soil environment in

the early industrial era was that generally held, rather optimistic concept of microbial infallibility (See chapter 4) combined with the confidence derived from the great knowledge that had been accumulated regarding the complexity of intermediary metabolism of soil bacteria and fungi. A portion of that trust in the capabilities of the soil microbes and the breadth of our understanding of their metabolic prowess was not misplaced. A vast array of biologically synthesized organic chemicals and industrially produced organic chemicals (xenobiotic chemicals) with structures that mimic the biologically synthesized substances are metabolized in soil. Of course, the full function of the microbial community is predicated on the precept that the proper environmental conditions must be maintained for optimal function of the soil community and that the pollutants do not enter the system at rates that require the microbial community to decompose them at rates faster than their maximum potential. More problematic are those xenobiotic chemicals that do not have structures resembling biologically synthesized molecules. These substances may be totally recalcitrant, metabolized in part (DDT conversion to DDE), humified (if sufficiently reactive chemically), or lost from the system through volatilization or leaching. Also, some of these chemcials may be slowly transformed cometabolically. For example, trichloroethylene (TCE) is decomposed cometabolically by microbes using methane or other simple carbon compounds as their carbon and energy source (See Wackett (1995) for a review of this topic.). Similarly, halogenated organic compounds can be dehalogenated cometabolically and the resultant product mineralized. A common example of this process is microbial reductive dechlorination of polychlorinated biphenyls (PCBs) (e.g., Bedard and Quensen, 1995). Approximately 1.4 billion pounds of PCBs have been released and have accumulated in the environment. In anaerobic environments the chlorine molecules can be removed from some of these substances thereby reducing the toxic risk of the contamination.

For many structurally complex organic chemicals, degradation relies on the existence of consortia or microbial assemblages to catalyze the processes. Microbial consortia are groups of microbial species that when acting together are capable of mineralizing a substance that no single member of the consortium can achieve alone. Perhaps the most commonly cited natural example of a group of members of the soil microbial community interacting to achieve complete mineralization of a complex substrate is the conversion of cellulose to methane. No single microbe is capable of mineralizing cellulose to methane; yet, in anoxic systems, methane production from cellulose is common. A variety of anaerobic bacteria (e.g., *Clostridium* spp.) convert the cellulose to the simple organic acids that are then utilized by the methanogens in their production of methane. Similar interactions are easily envisioned to occur in the decomposition of xenobiotic substances. The dehalogenation of PCBs described above can be seen as an initial step in a complex decomposition pathway of a seemingly environmentally stable

substance. A scenario can be described wherein the PCBs are dehalogenated under anaerobic conditions. The products then diffuse or are transported to an aerobic site where the biphenyl can be mineralized.

The best examples of exploitation of dehalogenation reactions for remediation of organic wastes are derived from studies of contaminated sediments, but there is no reason to anticipate that under comparable conditions such reactions would not occur in a soil ecosystem. A particularly interesting study is one in which terminal electron acceptors other than molecular oxygen were used for the degradation of monochlorobenzoate isomers by Nile river sediments (Kazumi et al., 1995). In this study, the monochlorobenzoates were degraded under denitrifying, methanogenic, iron reducing, and sulfidogenic conditions. Conversely, a study by Fliermans et al. (1988) demonstrated the capacity of aerobic microbial consortia to convert trichloroethylene (TCE) to hydrochloric acid and carbon dioxide. In the latter example, the consortia were isolated from subsurface sediments and grown in enrichment cultures. Other examples of microbial consortia based mineralization include decomposition of dicyclopentadiene (Stehmeier et al., 1996) and a complex mixture of aromatic compounds contained in olive oil mill effluent (Zouari and Ellouz, 1996). Similarly, in a complex biofilm, Field et al. (1995) demonstrated decomposition of aromatic pollutants in consortia of aerobic and anaerobic bacteria. Also, the anaerobic degradation of pentachlorophenol can be catalyzed by a methanogenic consortium (Juteau et al., 1995).

These examples demonstrate the variety of organic chemicals decomposed by microbial consortia and underscore the importance of the entire soil microbial community in bioremediation. This community or mixed culture per se involvement in bioremediation is particularly significant when it is considered that most situations wherein the indigenous soil microbial community is utilized to purify the systems, a mixture—generally complex—of organic compounds is encountered. Soils are rarely contaminated with a pure xenobiotic chemical. Even pesticides, which are commonly conceptually spoken of as if a single chemical substance were reaching soil, are commonly added to the soil-plant system in petroleum or other appropriate carrier mixture to aid dispersion and to facilitate dilution of the active ingredient to the appropriate dosage.

16.2.2.2 Role of Biofilms in Soil Remediation

This admixture of a variety of potential carbon and energy sources encourages development of interactive soil microbial communities. This observation leads to the question of the form or nature of the physical association of these microbes within the soil environment. As was indicated in chapter 2, bacteria exist in soil as discrete microcolonies whereas filamentous organisms may extend through several microhabitats. Much of the surface area of soil particles is not inhabited since the microorganisms tend to be concentrated in organic matter-rich areas. An

efficient microbial community assemblage which is commonly exploited for waste water purification (trickling filters) and fluidized beds is microbial biofilms. Biofilms are communities of microorganisms that develop at the interface of soil particles and water. They are primarily water (70 to 95 % wet weight) (Flemming, 1993) with the cells attached to the particle surface through extracellular polymers (70 to 95 % of the dry weight of the biofilm) (Flemming, 1993). Thus, the microbial cells are immobilized in a tightly organized structure, forming highly organized consortia. This physical arrangement is favorable for decomposition of complex mixtures of organic substances in systems with adequate or high nutrient levels and for optimal interaction of the community members (Costerton et al., 1995).

An additional consideration relates to the potential for a parallel conceptualization of the relationship of microorganisms in soils and the biofilms of trickling figures. That is, does a parallel community structure to the biofilm exemplified by trickling filters exist in soil and is it important for soil biological remediation. It is easy to envision the physical association of sessile microbes in soil within an assemblage akin to biofilms. The primary difficulty arises in considering the extent of the surface area of the soil particles occupied by such films. Soils tend to be carbon limited ecosystems that generally experience extremes in moisture, ranging from water saturated to highly xeric conditions. With a waste purification system, such as a trickling filter or a fluidized bed, optimization requires continuously flowing water (aerobic systems) or saturated water for those engineered processes in which anaerobic metabolism may be encouraged. These conditions (i.e., flowing oxygenated or deoxygenated water) generally do not exist in soil.

In spite of this observation, the biofilm concept can be invoked in part to explain the interaction of consortia in soil as well as the efficiency of water purification in systems such as wet lands (e.g., Alvord and Kadlec, 1996). Occurrence of highly developed biofilms is limited in most soil systems, at least for extended periods, due to the impact of biofilm development of saturated hydraulic conductivity. As the input of nutrients stimulates the development of the biofilm, the pore diameter is diminished sufficiently that saturated hydraulic conductivity can be rapidly reduced as much as four orders of magnitude (e.g., Vandevivere and Baveye, 1992), eventually reaching the point where flow of the nutrients into soil and maintenance of the biofilm community is precluded (Jennings et al., 1995). The rapidity of this biofilm development results from the tendency for colonization of the surface (inlet) of the soil column receiving the nutrient stream (see Vandevivere and Baveye, 1992). In highly structured soils, especially silt loams and clay soils, development of these pores results in the plugging of micropores. Thus, in high nutrient and water flow systems, a sandy matrix is more desirable to maintain the hydraulic conductivity (see Vandevivere et al., 1995).

Therefore, it can be concluded that the biofilm concept is useful in describing the micro-assemblages of communities of microorganisms on surfaces of soil particles as the result of locally elevated levels of nutrients. That is, a complex, interactive microbial community certainly exists in soil microsites. A more extensive macro-film, per se, could only be anticipated to occur under rare circumstances. The development of more traditionally described biofilm is limited to high nutrient situations, such as sites receiving chronic inputs of waste-laden waters, a situation less likely to exist with most bioremediation situations. For bioremediation, the microbes are encountering a varied distribution of the pollutant, some free in interstitial water (as would parallel the waste stream situation to some degree), some bound or sequestered within soil micropores. Thus, both the quantity and availability of the carbon and energy source would limit the physical size of the developing biofilm.

16.2.2.3 Importance of Substrate Concentration to Remediation Success

To this point, the key elements for microbial decomposition of organic pollutants considered include the following:

- The necessity of the microbe to posses the requisite genetic potential
- The ability to express that metabolic capability in a soil ecosystem
- The requirement for the microbe to possess the appropriate traits for successful interaction with other members of the soil biological community—including the ability to become at least temporarily established therein should the use of non-native organisms be required for successful site bioremediation
- The existence of consortia of microbial strains and species in assemblages or associations (biofilms), which optimize the capacity to decompose the complex mixtures of organic substances encountered in polluted soils which no single species or strain capable of removing the pollutant exists

A further biological trait to be addressed relates to the impact of the concentration of organic pollutant on the rate of microbial and enzymatic activities. For example, is there a concentration of the toxicant that inhibits or precludes the growth of the microbes or inhibits expression of the requisite enzymatic activities? There is an optimal range of pollutant concentrations that facilitates biological remediation. Thus, there is a need to assess our capability of managing the concentrations of these substances such that the quantities of pollutant are above the minimum required for induction and function of the enzymes required for their metabolism and below any threshold for toxicity to the decomposers. For example, the loading of the pollutant concentrations in the soil may require specific management. For example, two difficulties can be conceived to exist with excessive soil loading of toxic organic substances: a) The quantities of a carbon rich substance are sufficiently high that nitrogen or other nutrient limitation is imposed on the microbial

community and/or b) The pollutant is present at levels that are toxic to the decomposer community. In either situation, dilution or reduction of the toxicant concentration is the most easily conceived, but perhaps from an engineering viewpoint, less easily achieved. An example of toxicant loading reduction followed by biodegradation is exemplified by a feasibility study of remediation of hydrocarbon contaminated soil from beneath a paint factory (Origgi et al, 1997). In that situation, bioventing and biofiltration of the outlet gas stream resulted in significant remediation of the limited area contaminated with naptha, toluene and xylenes. The residual hydrocarbons were then reduced significantly through biodegradation.

A more challenging situation relates to systems containing extremely low levels of toxicants. An environmental or ecosystem risk may exist, but the concentration of the substance is below that generally required for active microbial mineralization to occur. In this situation, microbes capable of decomposing low concentrations of the pollutant must be enriched or the quantity of the substance encountered by the microbes raised to allow for biodegradation. The latter option emerges when high levels of pollutant exist in the system but the substance has low water solubility. Thus, from the microbes' view, only low levels of the essentially water insoluble pollutant are available for metabolism.

16.2.2.4 Managing Pollutant Water Solubility

With substances of limited water solubility, surface area is a primary determinant in controlling microbial transformations. A prime example of this situation is exemplified by the oxidation rate of elemental sulfur particles (see Chapter 14). Physical contact of the microbes with the sulfur is possible because the sulfur is not toxic. Thus, the microbes literally adhere to the sulfur particle surface. This situation is more complex when considering the decomposition of hydrophobic organic compounds. Growth on the surface of the crystalline organic may be precluded by its toxicity. Microbes existing at the surface of the crystal may actually be inhibited or killed by the capability of the hydrophobic organic contaminant to become associated with the hydrophobic portions of the cell membrane. One means of overcoming this solubility difficulty associated with hydrophobic organic substances is through the natural production of surfactants by the microbial community or use of soil amendment with industrially produced surfactants as an aspect of bioremediation plans.

Pertinent questions regarding the applicability of surfactants for the enhancement of bioavailability of soil organic contaminants of low water solubility include:

- Does the amendment of a contaminated soil with surfactants enhance the biodegradation rate of the organic pollutant?
- How does the degradation rate vary with surfactant type and concentration? That is, is more better? Are there advantages or disadvantages of anionic or nonionic surfactants?

- Are additional environmental problems created by the use of surfactants?
- If surfactant producing microbes are amended to soil, can they synthesize the surfactant and thereby enhance biodegradation of the pollutant of concern?

A variety of laboratory studies have been conducted with objectives of answering one or more of these questions. At this time, the general practicality of such research is still in question. A selection of representative studies will be examined to provide examples of the state of the art of such studies.

Evaluation of the efficacy of surfactants in the environment has been conducted using a variety of low water solubility substances, such as polycyclic aromatic hydrocarbons (PAHs), naphthalene, and xylenes, added to sediment and soil samples (e.g., Aronstein and Alexander, 1993; Grimberg et al., 1996; Liu et al., 1995; Zhang et al., 1997). In each situation, it was generally concluded that the amendment of the hydrocarbon contaminated solid matrix with surfactants stimulated the biodegradation rate of the water insoluble contaminant. Some effect of surfactant usage on both organic and inorganic soil contaminants has been noted to occur. Huang et al. (1997) found that addition of an anionic surfactant to a soil containing both lead and naphthalene resulted in enhanced solubilization of the naphthalene and desorption of the lead ion, suggesting that the surfactant could be used to increase mineralization of the hydrocarbon and to enhance the capacity to leach the lead from the soil profile.

Surfactant concentration is a significant concern for reclamation enhancement in that as its concentration increases so does the tendency to form micelles. The impact of micelle formation is that the hydrophobic pollutant may become stabilized in the micelle and thus remain unavailable to the microbial community. This was shown by Volkering et al. (1995) in their evaluation of the utility of several nonionic surfactants for enhancing the solubility of phenanthrene in batch growth experiments. In contrast, Liu et al. (1995) found that naphthalene solubilized by micelles of two nonionic surfactants was bioavailable to a mixed culture of bacteria. Zhang et al. (1997) found that phenanthrene within micells of the nonionic surfactant monorhamnolipid was less bioavailable than that contained within dirhamnolipid micelles, suggesting variation in the bioavailability due to the nature of the surfactant forming the micelles. Vanhoof and Jafvert (1996) noted that dechlorination of hexachlorobenzene varied with relationship of the surfactant level to the critical micelle concentration. For example, Tween 80 decreased dechlorination at concentrations significantly above the critical micelle concentration, whereas stimulation was noted at or below the critical micelle concentration.

Further concerns with using high concentrations of surfactant relate to the potential for toxicity of the surfactant and impact on the biological system due to the degradation of the surfactant itself. Toxicity of the surfactant is readily

determined through evaluation of the effect of amendment of soil with varying concentrations of surfactant on key indicators of microbial activity, such as respiration or even mineralization of the toxicant of interest. The effect of biodegradability of the surfactant is perhaps more interesting. A study demonstrating both toxicity of surfactants and the effect of biodegradation was presented by Tiehm (1994) where it was noted that toxicity of the surfactants examined decreased with increasing hydrophobicity. The non-toxic surfactant were effective in enhancing the degradation of a variety of hydrocarbons, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene. Interestingly, sodium dodecyl sulfate inhibited the degradation of the polycyclic aromatic hydrocarbons because it was a preferred substrate. In a related study (Tiehm et al., 1997), biodegradable surfactants were found to interfere with surfactant enhancement of biodegradation of polycyclic aromatic hydrocarbons in manufactured gas plant soil due to depletion of molecular oxygen during mineralization of the surfactant.

Surfactants can be synthesized by soil microbes. Thus, it could be proposed that it could be advantageous to amend soil, not with the surfactant itself, but rather add a microbe that could survive, grow, and produce the surfactant in situ. An example of this type of system was described by Barriault and Sylvestre (1993). They amended soil contaminated with Aroclor 1242 (PCBs) with a PCB degrading bacterial strain, biphenyl as a cosubstrate to stimulate PCB degradation, and a second bacterial strain, *Alcaligenes faecalis* strain B-556, which produced a surfactant. Decomposition of the Aroclor was stimulated over that not receiving the surfactant producer.

Use of soil inoculation to stimulate bioavailability of water soluble toxicants is more complex (beyond the regulatory difficulties) than simple amendment with the surfactant since a) the microbial strain producing the surfactant must be capable of survival and function within the soil system, and b) the surfactant must be produced in effective concentrations at the site where it is needed. That is, the surfactant synthesizer, the target pollutant, the organisms responsible for its decomposition, and the surfactant must all co-exist in the same microsite at appropriate levels for optimal activity. If the decomposer population is not physically associated with the pollutant and the surfactant, then the solubolized pollutant must diffuse or be leached to the location within the soil matrix where the biodegradation can occur.

16.2.2.5 Biological Remediation of Soils with Low Loading of Pollutant

The above analysis of the complexities involved with the use of surfactants is predicated on the assumption that the soil site has been contaminated with reasonably high levels of the pollutants but that the bioavailability to the decomposer community is low due to their low water solubility. Situations may also exist where the concentrations of the organic chemical in interstitial water is limited

simply because the pollutant is present at low levels. For this consideration, a low level of a pollutant is considered to be one for which there is environmental concern but the organic compound is present at levels that are insufficient to serve as a carbon and energy sources for soil microbes. In this case, the primary limitation to mineralization results from the probability of encounter of the decomposable substance and members of the decomposer population or their extracellular enzymes and the capability of the requisite enzymes to catalyze the process. The latter situation results from the fact that enzyme efficiency is optimal at substrate concentrations approximating the Michaelis constant (K_m) of the enzyme. These levels are generally several orders of magnitude greater than the quantities of substrate considered in this discussion.

If the metabolizable substance is present in concentrations below that which can effectively react with the enzyme, then its stability in soil will mimic that of a recalcitrant substance unless mechanisms exist that will result in elevated concentrations in microsites. One such commonly encountered mechanism in soil results from sorption on clay surfaces. Two processes are involved in this situation. The biodecomposable pollutant is concentrated on the clay surfaces so that an elevated level of the substance exists in relationship to the soil surrounding the microsite. But, this material, due to the fact that it is sorbed on the clay surface is of limited availability to the microbial community. Since sorptive processes are reversible, this sorption of the pollutant on the clay surface also results in augmented levels in the water surrounding the clay particles. Thus, the microbe occupying the microsites, should it be capable of decomposing the pollutant, will mineralize the pollutant resulting in a reduction of the soil loading of the pollutant below levels that would be generally predicted from assessment of the pollutant concentration.

A further mechanism that can result in augmented mineralization of low level organic pollutants in soil involves stimulation of growth or activity of the decomposer population through amendment of the soil with alternate growth substrates. As was indicated above, a low level contamination of soil is defined as one in which the substance is present in concentrations less than those that are necessary to sustain microbial growth. Thus, the situation where the both the population of decomposers and the substance to be decomposed are sufficiently low that their encounter is improbable. Augmentation of the microbial population due to the presence of the pollutant can not be anticipated due to the fact that it is present in concentrations below that needed to serve as carbon and energy source. An alternative in this situation is to amend the soil with a metabolizable substance that increases the general soil microbial population (e.g., Pahm and Alexander, 1993; Schowanek et al., 1997). Schowanek et al. (1997) amended soil with tetradecenyl succinic acid (TSA), a major component of a detergent builder, with and without sewage sludge. Decomposition of the low level of TSA in the

absence of sludge was preceded by a 13 day lag period whereas no lag was observed when it was accompanied by sewage sludge. Similarly, Pahm and Alexander (1993) were able to stimulate the mineralization of low concentrations of *p*-nitrophenol (PNP) in soil amended with PNP degrading bacteria by the addition of glucose. Presumably, in each situation, the enhancement of the energy supply to the microbial community by the glucose and sewage sludge resulted in a sufficiently augmented energy supply for the microbial community that the pollutant concentrations could be gratuitously reduced by microbial mineralization.

16.2.3 Microbial Inoculation for Bioremediation

Given the conclusion that a soil microbial community continually adapts to its changing, evolving environment, it is logical to conclude that new members of the community are continually being recruited. These alien populations may arise from propagules that enter the soil ecosystem on soil particles carried by wind or in water or on the surfaces of invading roots. Thus, we can logically conclude that adaptation of a microbial community to chemical pollution through invasion of alien organisms should, or even must, occur, especially if soil adjacent to the contaminated site contains the appropriate decomposer populations. Therefore, the primary question regarding management of bioremediation of soil systems is not whether the potential for adaptation of the native communities to the contamination is possible. The composition of the soil microbial community must be resilient to insure survival of the ecosystem as environmental conditions evolve. The perhaps more pertinent question is: can the community development be manipulated so that highly efficient decomposer populations are selected? A logical extension of this question is: Can laboratory developed microbes (including those genetically engineered) amended to a soil system become part of the functioning soil microbial community catalyzing the effective and efficient renovation of the site?

The literature is replete with reports supporting the potential use of a variety of bacteria and fungal species for bioremediation of soil (e.g., see Madsen and Kristensen, 1997; Miethling and Karlson, 1996; Sack and Fritsche, 1997; Smith et al., 1997 as recent examples of such studies). This amendment of soil with decomposer populations may also be supplemented with a selective substrate to facilitate establishment of the inoculated population (Lajoie et al., 1993; Nishiyama et al., 1993) in situations where the host receives no or minimal advantage from the mineralization process. Furthermore, Madsen and Kristensen (1997) provide an example where a surfactant is introduced with the exogenous bacterial population to stimulate decomposition of an organic contaminant. In this latter example of introduction of microbial strains or species into a soil, polycyclic aromatic hydrocarbon decomposition was stimulated by enhanced availability of the

hydrocarbon due to interaction with the surfactant produced by the laboratory cultured microbial strain. Another interesting example of such research involving managing the soil biological community for optimization of soil remediation was conducted by Siciliano and Germida (1997). They suggest that both the plant and the microbial community can be managed to enhance bioremediation.

16.2.3.1 Rhizosphere Management for Biological Remediation

When considering the interaction between plants and soil microbes from the view of enhancing bioremediation potential, the unique properties of the rhizosphere must be emphasized. Recall that the rhizosphere soil is that portion of the ecosystem where biological activity is maximized (See chapter 8). Thus, it can be generally concluded that mineralization reactions are maximized therein. Linked with the above cited studies demonstrating enhanced decomposition of organic pollutants by addition of metabolizable organic matter, the conclusion that bioremediation processes should be enhanced in the rhizosphere is logical. The question thus becomes: Are there data suggesting benefits of rhizosphere interactions in bioremediation?

A variety of studies have suggested augmented decomposition of agricultural chemicals and nonagricultural chemicals in rhizosphere soils (See Anderson et al., 1993, for a review of this topic.). For example, Reilley et al. (1996) found that plants can enhance the decomposition of polycyclic aromatic hydrocarbons in the immediate environment of the root. Their study suggested that once the toxicity of petroleum components to plants has been sufficiently reduced, incorporation of plants into a landfarming scheme can augment the bioremediation effectiveness. This stimulated activity can result simply from the general elevation of the microbial populations in the rhizosphere due to the energy and nutrient supply of the root exudates (e.g., Bollag et al., 1994). Gilbert and Crowley (1997) also note that some plant-derived terpenoids may also promote polychlorinated biphenyl degradation by an *Arthrobacter* strain in the rhizosphere.

Considering that there are numerous studies demonstrating that ectomycorrhizal fungi are capable of mineralizing complex organic compounds, it is also logical to conclude that mycorrhizal associations may also be beneficial for bioremediation (e.g., Gaskin and Fletcher, 1997). For this benefit to be maximized in a managed ecosystem, propagules of the mycorrhizal fungus must be present (either through plant inoculation or as indigenous soil populations) and the plant community must develop sufficiently for these symbiotic associations to form.

A complication that must be considered when managing both microbial and plant communities for bioremediation is the potential for the formation of plant toxic intermediates during decomposition of the pollutant (e.g., Hoagland et al., 1994; Pfender, 1996). Bioremediation potential of the microbe-plant system may be lost if toxic intermediates, which are not further metabolized, accumulate in the rhizosphere.

16.2.3.2 Distribution of Alien Organisms in Soil

In the studies cited above, the primary concern of the research was whether the organisms were capable of decomposing the target organic compound within a soil matrix. That potential activity has been adequately demonstrated in test tube studies. The next question relates to the capability of applying laboratory selected microbes to soil in a manner that allows them to pass through soil pores to the site where their action is required.

In a fluidized bed reactor, it is reasonably easy to introduce an alien microbial strain and to reach the conclusion that through the mixing process of the reactor the organism will encounter the target organic compound and mineralize or detoxify it. This capability of transporting the microbe to the site of requisite action in soil is more problematic. Generally, maintenance of as much of the native soil structure during reclamation of a soil ecosystem is desirable. Also, due to the large area requiring reclamation, mixing of microbial populations throughout the contaminated profile is generally impractical. Thus, the dynamics of microbial movement within the highly structured soil environment becomes a concern for evaluating the utility of soil inoculation for bioremediation. Considerable research has been conducted regarding the transport of various microbial strains, primarily within sand columns, and a variety of mathematical relationships have been developed describing the various transport phenomena in a variety of soil systems (e.g., see Baveye et al., 1998; Devare and Alexander, 1995; Murphy and Tate, 1996; Natsch et al., 1996). The primary conclusion from these studies is that bacteria can be transported through soil macropores preferentially during saturated flow, such as after a heavy rainfall. Limitations relate to plugging of soil pores when high populations of microbes are amended to the soil and the inability of the amended microbes to enter micropores due to size constrictions. Thus, although it can be demonstrated that with proper consideration of inoculum density and flow dynamics bacteria may be added to soil and distributed therein, the bioremediation accomplished by such processes is limited by the capability of the organisms to penetrate all microsites of the soil profile containing the pollutant. The microbes can easily be envisioned to pass through and colonize macropores and some micropores, but pollutant contained within the smaller micropores would be inaccessible to the added microbial cells.

16.2.3.3 Survival of Alien Microbes in Soil

A further concern associated with utilization of laboratory selected microbial strains for bioremediation relates to their survival beyond the time necessary for renovation of the site. It is generally considered desirable for the alien population to be eliminated once the pollutant has been exhausted or diluted to an acceptable level. Generally, to enhance biological containment of genetically modified organisms within soil systems, it is desirable to limit the survival of the organisms

without limiting their beneficial effects. This process can be accomplished through the use of controlled suicide systems (e.g., Jensen et al., 1993; Knudsen and Karlstroem, 1991; Molin and Kjellber, 1993;). For example, Jensen et al. (1993) describe the utilization of two element suicide system for the containment of *Pseudomonas putida* KT2440. Each genetic element of the suicide system operated independently to enhance the population decline once function of the microbe was no longer required. Ahrenholtz et al. (1994) describe a system where the cell population is regulated through the destruction of intracellular DNA available for horizontal gene transfer processes.

Allied with this use of genetically engineered microbes with optimized decomposition capabilities and diminished extended survival potential (post depletion of the organic pollutant) is a consideration of our capacity for detection of the microbes. Highly sensitive techniques are required to assure detection of low populations of the amended organisms. A useful, but less sensitive, procedure involves utilization of the BIOLOG method for assessing metabolic diversity (See chapter 3). Vahjen et al. (1995) evaluated the use of this procedure for detection of *Corynebacterium glutamicum* in inoculated with 10^6 colony forming units g^{-1} soil. They were able to detect the presence of the inoculated organisms as long as the cell density was greater than 10^5 colony forming units g^{-1} soil. Matheson et al. (1997) have provided an example of using DNA probes to detect the persistence of a particular strain of *Berkholderia cepacia* when as few as 10 colony forming units occurred in a population of 10^5 non-target colony forming units. Generally, DNA based probes are preferable for assessing survival of exogenous microbial populations in soil ecosystems.

An interesting application of genetically modified microbes in soil remediation is incorporation of fluorescence genes into the bacterial genome to provide an indication of the bioavailability of the organic pollutant within the soil system and of the degradation of the pollutant (Burlage et al., 1994; Heitzer et al., 1994; Rice et al., 1995; Webb et al., 1997). For these studies, reporter strains of bacteria are developed through incorporation of the bioluminescent lux genes from *Vibrio fischeri* into the genome of the genetically engineered microbe. The genes are transcriptionally fused to the catabolic gene sequence so that they are expressed whenever the catabolic genes are expressed. That is, exposure to the contaminant results in inducible bioluminescence. The magnitude of the response and the response times were shown to be concentration dependent in a system developed with *Pseudomonas fluorescens* for the decomposition of naphthalene and salicylate (Heitzer et al., 1994). Thus, these organisms serve as *in situ* indicators of the bioavailability of the pollutant (if it is present but not bioavailable, the bioluminescence genes are not induced), decomposition of the pollutant (the catabolic genes are synthesized concurrent with the bioluminescence genes), and the termination of decomposition (the genes are no longer expressed).

16.3 Soil Properties Controlling Bioremediation

The impacts of the soil physical environment on bioremediation outcome can be grouped into two categories; factors that delimit biological activity and properties that limit accessibility of the decomposer populations to the pollutant (i.e., sequestration of chemical pollutants). General analysis of the physical and chemical requirements of the soil microbial community was presented in Chapter 5. Thus, the discussion here will be limited to specific examples of ecosystem management to optimize a particular biological transformation or process.

16.3.1 Physical and Chemical Delimiters of Biological Activities

The primary soil properties managed for bioremediation considerations are soil pH (generally for the control of metal mobility), nutrient supplies (macro- and micronutrients as well as carbon and energy sources), and soil moisture/aeration. These delimiters of expression of biological activity provide key examples of how the rate, nature of the process, and ultimate fate of the pollutant can be selected by managing the soil physical environment. Due to the resilience of the soil microbes, fully functional microbial communities are found in what could superficially be considered to be impossible conditions. Therefore, an initial planning question for biological remediation becomes: Are the soil conditions conducive for development or existence of a fully functional microbial community? If this question is answered in the affirmative, then concerns relate to specific effects of the physical and chemical environment on the decomposition of the substance of concern (especially the biochemical processes involved) and the bioavailability of the pollutant. If not, the prevailing conditions which preclude life must be modulated prior to implementation of a management plan for control of the microbial community to optimize bioremediation.

16.3.1.1 pH

In the previous consideration of the effect of soil pH on microbial activity (Chapter 5), it was noted that microbial growth and activity occurs in both acidic and alkaline soil ecosystems. Therefore, there is no inherently bad implication of the occurrence of soil systems at the ranges of pH values commonly beyond the ranges normally encountered for agricultural production in temperate regions (slightly acidic to neutral pH). In fact, numerous tropical cropping systems (pineapple, cassava, etc.) are productive on soils with pH's of less than 4. The major requirement for reclamation management is that the full implications of pH on the processes occurring in the chemically polluted soil be considered. Examples of soil processes where pH effects need to be evaluated are nutrient cycling and effects of pH on the physical and chemical states of the organic chemical of question.

Nutrient cycling processes most commonly managed for bioremediation relate to nitrogen availability. Note that it was indicated in chapter 11 that both nitrification and symbiotic nitrogen fixation can be impacted by acidic conditions. Autotrophic nitrifiers function above pH 5.0 to 5.5 whereas the *Rhizobium-legume* symbiotic associations for nitrogen fixation require a soil pH greater than about 5.5. Thus, for example, in acidic soils with low levels of fixed nitrogen, the soils will need to be limed to a pH level conducive for nitrogen fixation to occur with legumes, or actinorhizal associations, such as bayberry, have to be cultivated.

The greater concern involving soil pH is likely the impact of pH on the ionic state of the pollutant and the resultant effect of this charge variation on its interaction with the enzymes involved in its mineralization or detoxification as well as its sorption to soil organic matter and clays. At pH values above the pKa, acidic groups are ionized, facilitating ionic interactions with enzymes as well as the physical environment whereas below the pKa, hydrophobic interactions are favored. Similarly, from an environmental health view, hydrophobic interactions tend to favor associations with membrane lipids and bioaccumulation of the toxicants. An example of the effect of pH variation on the state of an organic chemical in soil is provided by looking at the sorptive properties of atrazine and fomesafen, two pesticides, in soil (Weber, 1993). Decreasing pH resulted in higher sorption of both herbicides. Atrazine sorption resulted from ionic bonding at low pH and physical bonding at neutral pH whereas fomesafen sorption was the product of physical forces at neutral pH and hydrophobic bonding and/or precipitation at low pH. Thus, at lower pHs, the bioavailability of the pesticides could be anticipated to be less than at high or neutral pHs.

16.3.1.2 Nutrient Amendment and Bioremediation

Examples of nutrient amendments for bioremediation of soils are generally associated with hydrocarbon rich contaminants, such as petroleum. It has long been known that nitrogen fertilization is necessary to stimulate petroleum degradative with land farming. Even in situations where other variables such as temperature in alpine soils (Margesin and Schinner, 1997) or salinity and temperature with beaches of Prince William Sound (Pritchard, 1991), use of nitrogenous fertilizers enhanced biodegradation. Other nutrient amendments may also involve provision of a carbon and energy sources in situations where the pollutant can only be decomposed cometabolically (e.g., carbon tetrachloride and a variety of easily metabolized carbon sources (Lewis and Crawford, 1993)) or simply from the general stimulation of the microbial population (as indicated above). In the latter situation, composted waste materials provide an energy source for the general soil microbial populations (thereby stimulating mineralization of the contaminant), stimulate soil aggregate forming processes resulting in an improved environment for function of the microbial community, improve the soil moisture dynamics of the system, and also result

in increased humification of the organic toxicant, thereby providing an alternative path for removal of the toxicant from the system (e.g., Kastner et al., 1995; Laine and Jorgensen, 1996). Thus, recycling of composted societal by-products can assist in optimization of soil bioremediation processes.

16.3.1.3 Soil Moisture/Aeration

As was indicated in Chapter 5, the world of soil microbes can be conceptualized as an aquatic system, albeit a thin film of water covering a largely particulate matrix. The organisms are bathed in this film of water, nutrients are transported into and away from the cell by diffusion or mass transport processes, and the water itself is a requisite participant in many biochemical transformations. These observations are true whether we are dealing with a native soil ecosystem or a remediation site. Of particulate concern in managing polluted soil is the interrelationship between soil moisture and soil oxygen status. That is, decomposition rates, detoxification processes, the extent of mineralization, and the nature of the metabolic intermediates accumulating vary with the nature of the terminal oxygen acceptor. Much bioremediation philosophy has been predicated on the objective of optimizing free oxygen levels. This management strategy is based on the assumption that oxygen based metabolism is more rapid and generally leads to accumulation of fewer metabolic intermediates than would occur under anoxic conditions. Considering that the terrestrial system is bathed in an oxygen rich atmosphere, this philosophy is generally reasonable, although as will be indicated below, consideration of other terminal electron acceptors (e.g., nitrate, sulfate, carbon dioxide) is useful in naturally anoxic systems where manipulation of aerobic processes is impractical.

The examination of native soil ecosystems, such as swamps and related water saturated systems, leads to a conclusion that organic matter decomposition is slower and the accumulation of partially decomposed organic intermediates is accentuated in such systems. It has long been known that the versatility of the soil microbial community in dealing with reduced oxygen levels is revealed in such systems. For example, reduction of oxygen tensions a percent or two from the normal atmospheric level of approximately 21.0 % is limiting for higher animals, but in drained histosols, microbial mineralization of the accumulated organic matter continues to free oxygen tensions of less than 1 % (see Broadbent, 1960). Similar relationships of biodegradation processes to available oxygen levels would be anticipated to occur in soils contaminated with organic chemicals, although the rate of decomposition will necessarily vary directly with oxygen tension as the oxygen levels become limiting. Examples of limitations to bioremediation by oxygen levels are common in studies of soil and sediment remediation processes (e.g., Hurst et al., 1997; Madsen et al., 1996). Hurst et al. (1997) noted that pentachlorophenol degradation in contaminated soil from a superfund site was precluded in the absence of oxygen, but they were enhanced by soil oxygen concentrations between

2 and 21%. They concluded that a soil oxygen concentration of at least 2 to 5% was necessary for pentachlorophenol bioremediation. When depletion of soil oxygen levels by biological activity exceeds the diffusion rate for oxygen replenishment, the oxygen tension can be elevated through bioventing (e.g., Hurst et al., 1997) or even amendment of the soil with peroxides (e.g., Fiorenza and Ward, 1997).

In some situations, soil oxygen can be depleted to optimize processes favored by anoxic conditions (e.g., dehalogenation or reduction of nitro groups). Kaake et al. (1992) describe a soil treatment method for removal of the pesticide dinoseb (2-sec-butyl-4,6-dinitrophenol) where soils were pretreated by amendment with a starchy potato processing by-product to reduce the redox potential and create anaerobic conditions. An anaerobic microbial consortium capable of mineralizing dinoseb without formation of polymerization products commonly seen under aerobic or microaerobic conditions developed. Alternatively, alternation of aerobic and anoxic conditions can be used to facilitate bioremediation. For example, a two stage process starting with an anaerobic conditions facilitates the bioremediation of munitions compounds including 2,4,5-trinitrotoluene (TNT) (e.g., Funk et al. 1993; Breitung et al. 1996). During the first phase, ammunition residues and aminoaromatic compounds were depleted and the subsequent reduced products were mineralized during the aerobic phase of the process.

It is a given that the bulk of organic matter decomposition, by far, in soil results from oxygen based processes. Thus, it is logical that initial considerations in developing bioremediation plans must involve evaluation of the potential for optimization of aerobic soil metabolism; but, there are many contaminated soil ecosystems where utilization of alternate electron acceptors is more practical and economical due to the fact that the site is naturally anoxic. A further encouragement in this consideration is the fact that in many instances the soil contaminants may include nitrate or sulfate, two logical terminal electron acceptor alternatives. Furthermore, dissimilatory nitrate and sulfate reduction yield energy recoveries to the microbes approximating those gained from oxygen metabolism. Thus, it is reasonable to conclude that these processes should be as efficient and rapid as the more commonly studied and applied aerobic degradation processes.

Denitrification is the most studied anoxic process for bioremediation. Denitrification, as a means of reducing soil nitrate loading, by definition is a bioremediation process (e.g., see Smith et al., 1994; Weier et al., 1994). Since a carbonaceous compound is generally mineralized as the source of electrons for denitrification, a potential exists for selection of denitrifiers that mineralize toxic organic compounds for bioremediation of soils contaminated with both nitrate and decomposable organic chemicals (see Casella and Payne (1996) for a review of this topic.) Indeed, as indicated above, a variety of potential soil toxicants are mineralized by denitrifiers (see also Crawford et al., 1998; Haner et al., 1995). A major question remaining relates to the capability of managing bioremediation in

field situations under denitrifying conditions. An example of the potential for manipulation of anoxic processes is provided by bioremediation of BTEX (benzene, toluene, ethylbenzene, and xylene) in petroleum contaminated soils. BTEX degradation has been noted with ferric iron, sulfate or nitrate as terminal electron acceptors as well as under methanogenic conditions (Lovley, 1997). Denitrification alone is apparently not sufficient to bioremediate gasoline (BTEX) contaminated soils due to the inability of the denitrifiers to decompose benzene (Kao and Borden, 1997).

16.3.2 Sequestration and Sorption Limitations to Bioavailability

A real limitation to the successful conclusion of biological remediation efforts relates to the quantity of residual pollutant remaining in the system. The longer the time interval since initial contamination of the soil, the greater the probability that significant quantities of the organic pollutant will not be accessible to the biological community. A key to understanding this limitation to the ultimate cleanup of the site resides in developing an understanding of the impact of soil heterogeneity on the biological processes occurring therein. As was emphasized in Chapter 1, the variety of minerals, complex organic substances, as well as biological entities existent in any soil results in a myriad of different microenvironments within which soil microbes function. This heterogeneity affects both the immediate and potential bioavailability of decomposable organic substances and toxicants. For an organic compound to be decomposed or for this substance to inhibit microbial activity, it must have contact with the microbe or enzymes produced by the microbe. (Recall that extracellular enzymes may transform organic matter into water soluble products, which through mass transport or diffusion come into direct contact with the microbial cell.) Two primary properties of the soil system that affect bioavailability are physical location within the soil pore structure and sorption onto soil particulate surfaces.

Due to the dynamic nature of the distribution of materials within the soil matrix, the quantity of substance sequestered within soil pores, sorbed to soil minerals or dissolved in soil water changes with time. It is commonly observed that the bioavailability, chemical extractability, and decomposition rate of soil organic contaminants decreases with time since initial exposure (e.g., Alexander, 1995; Grant et al., 1995; Radosevich, 1997). This time dependent variation in bioavailability can be demonstrated through assessment of percent uptake by earthworms, bacterial degradation, or solvent extractability (e.g, Kelsey et al., 1997). For example, Kelsey and Alexander (1997) found that less rigorous solvent extraction methods correlated better with the availability of atrazine, phenanthrene and napthalene to earthworms in sterile soil than did the more vigorous extraction procedures commonly used to assess soil contamination. The degree of

sequestration varies with time as well as with soil properties, such as clay and organic matter contents (e.g., White et al., 1997). Furthermore, the source of the microbial inoculum may also affect the extent of biodegradation that occurs in the soil system. Sandoli et al. (1996) found that microbes isolated from their sand sediments that were contaminated with phenthrene decomposed the contaminant with a shorter lag period than did those from other sources.

These observations support the hypothesis that the bioavailability of organic substances in soil to decomposition by soil microbes relates to the pore structure of the soil as well as the capacity of the substances to interact with clay minerals and colloidal organic matter. This hypothesis has been supported by use of model systems composed of glass, polystyrene, diatomite, and silica beads with varying pore structure and hydrophobicity (Nam and Alexander, 1998). This study demonstrated that both hydrophobicity and existence of nanopores in the solid matrix affected the bioavailability of phenanthrene.

It is reasonably easy to envision the nature of the effect of micropore structure on chemical-microbe interactions. Substances trapped within soil pores of mean diameters less than that of microbes and containing no microbes would not be detoxified or mineralized. The quantity of pollutant retained in micropores is controlled by the physical forces dictating soil water dynamics. Entrance into the pores is diffusion limited, as opposed to the soil macropores where saturated flow allows for mass transport of solutes in gravitational water. Once solutes enter the micropores their exit from the system is controlled by a) diffusion, b) their retention within the more rigid crystalline water layer on the pore surface, and c) their tendency to sorb onto the surfaces of clay or organic matter surfaces in the pores. The role of sorptive phenomena is documented by the observation that biodegradation in soil containing both pollutant and its decomposers is frequently proportional to the desorption rate of the pollutant (Carmichael et al., 1997). The fact that the nature of the mineral component of the soil (especially clay type) affects decomposition kinetics underscores the need to evaluate the soil mineralogy (e.g., Appitz and Meyersschulte, 1996; Handerlein et al., 1996). Non-covalent interactions of organic substances with soil humic substances can result in associations with stability resembling that of covalently bound substances (Dec and Bollag, 1997). Chemical models have shown that simple organic compounds can form stable associations with clay surfaces and within pores of soil humic acids that are sufficiently stable to essentially remove the toxicant or biodegradable substance for the biologically available nutrient pool (Schulten, 1995; Schulten and Schnitzer, 1997). Thus, bioavailability of organic chemicals in soil and their potential for biological remediation is controlled by the tendency of the substance to diffuse into and out of soil micropores as well as by the forces preventing diffusion of the chemical back out of the pore (sorption, non-covalent stabilization in humic acids and retention in crystalline water.)

16.4 Concluding Observations

Bioremediation of polluted soils is only a possibility because of the extensive diversity of the soil microbial community and the redundancy of capabilities that exists therein. Indeed, even though there is a potential to develop laboratory derived microbial species and strains capable of decomposing soil pollutants in situ, the bulk of soil biological remediation is facilitated or accomplished by the indigenous soil microbial community. Thus, success of any remediation project relies totally on a clear understanding of the biological capability inherent in the system and the physical, chemical, and biological limitations of the expression of that activity. A variety of soil properties can be varied to optimize the rate and extent of biological remediation, but the ultimate controller of the quantity of toxicant remaining in the system is its bioavailability. Retention of chemicals within soil micropores and association with soil minerals and organic matter precludes total elimination of the soil contaminant. The ultimate environmental risk of this residual toxicant is determined by the potentiality for the bioavailability to change with time. The primary unanswered question in relationship to the overall risk of retaining sequestered toxicants in a remediated soil relates to the still to be determined probability that the toxicant will be released in meaningful concentrations at a future date. Based on the above analysis of the limitations to bioremediation, a meaningful concentration would be one that poses a toxic threat to the function of the indigenous biological community or to higher plants or animals in the ecosystem.

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Concluding Challenge

In the preceding pages, the multifaceted world of soil microbes has been revealed. It is not difficult at this point of our study to reach the conclusion that each of the individual habitats of soil microbes is nearly unique. Microsites are composed of a diversity of physical components assembled in varying degrees of structure ranging from an apparently structureless array of sand grains to the complex, ever-changing combinations of mineral and organic matter termed soil aggregates. Furthermore, microbes encounter an infinite combination of soil organic compounds ranging from simple organic acids, amino acids, and monosacharides to the highly complex humic acids. This assemblage of mineral and physical components of the microbial world is further varied by the presence of a variety of inorganic substances, many of which provide the energetic basis for growth and development of a portion of the soil microbial community known as the chemolithotrophs. An additional layer of complexity of the soil system is provided by myriad potential members of the microbial community and the variations in their interactions. The complexity of the soil biological community is only partially revealed by this abbreviated illumination of the microbial world. Growth and development of individual microbial cells and the associated alteration of soil components through cellular energy metabolism and the by-products of this activity appear to be nearly chaotic.

In contrast, microbial growth in test tubes is highly ordered and easily described by a variety of somewhat simple mathematical relationships. In soil, a highly productive cell may only be allowed one to a few cellular divisions before the stresses of space, nutrients, or biological interactions reduce the proliferation rate. In reality, it is difficult to envision a situation in soil where the maximal division rates commonly seen under the optimal conditions used in laboratory investigations could ever occur. Further, quiescent organisms may suddenly be spurred into a few rounds of seemingly unimpeded division by the influx of an energy source or temporary relief from growth-inhibiting conditions, but this time of luxury can

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just as quickly be disrupted by exhaustion of the newly found growth sources or imposition of new impediments to proliferation.

The preceding chapters described the basic principles of the discipline of soil microbiology that provide some order for this highly disordered world in soil and for prediction of the behavior of the soil microbial community as the properties of their habitats are altered. With our continually growing appreciation of the complexity of the soil microbial community and the interactions between its members, it becomes readily apparent that the information revealed in these pages only serves as a guidepost along the path to a more complete understanding of the home of the soil microbes and the genetic potential contained therein. This pathway could be likened to a divided highway leading to the ultimate in understanding and wisdom in this discipline. The seemingly contradictory endeavors of delving into the minutiae of the system must be combined with just as diligent a drive to conceptualization of the role of the soil microbes in function of the total ecosystem. This latter system must extend far beyond the simple soil of a meadow or mineland reclamation site to the totality of terrestrial systems and perhaps beyond.

As our capacity to understand, and perhaps model, larger landscape units increases, our intellectual concept of an ecosystem has enlarged. Instead of being a limited area, reasonably homogeneous from the perspective of aboveground aspects, we are becoming more aware that a true ecosystem is a mixture of contiguous but clearly distinct systems (e.g. a swamp bordering a forest system) as well as a continuum of slowly changing systems. Nowhere is this concept of an ecosystem as a complex mixture of interacting system types more clearly demonstrated than in soil, where we find subsoil processes affecting surface soil systems that are further regulated by external inputs such as organic matter and moisture from the aboveground portions of the grander system. On a microscale, we can also develop a realistic conceptual scenario of complex interactions of a variety of microsites being summed to produce the product that we may describe as a particular soil system, such as a grassland.

With this more inclusive and less reductive view of ecosystem description comes an enhanced appreciation of the impact of soil microbiological processes on total terrestrial processes. Necessarily, the basic principles described in the preceding chapters were elucidated with examples of limited ecosystem types, sometimes simply the system that develops from a soil sample contained in a test tube. Thus, a somewhat disparate view of the impact of such processes as denitrification (and associated nitrous oxide production) on general terrestrial processes can be gained. It is easy conceptually to separate the internal portions of the soil aggregate wherein denitrification occurs from the external oxygenated regions of the same aggregate. More dramatically, conceptually, the denitrification occurring in a swamp, or even a constructed wetland, can readily be isolated from its impacts

on the function of the surrounding systems, be they forest, agricultural lands, or even urban environments.

Taken in isolation, the concepts of soil microbiology could almost be considered to be evolving at a "snail's pace," yet once we move our mental contemplation from the limited world of the specific microbe or process, a multitude of major challenges to the environmental microbiologist arises. Concerns such as predicting the impacts of global warming and the role of soil microbes as they interact with the organic resources on the product of greenhouse gases, maintenance of soil quality, not to mention the problems of reclaiming chemically polluted soils or soils damaged from mismanagement, demand an expansion of soil microbial concepts further than ever before. Solutions to these environmental problems and support for efforts such as global climate modeling can be derived to some degree from extrapolation of data and concepts derived from studies of more limited ecosystems. But the greater challenge to soil microbiologists resides in developing reliable models and data sets supportive of more general models of the total ecosystem (the entire terrestrial system), predictive of the impact of anthropogenic activities and of our reclamation efforts.

This treatise was introduced with the objective of presenting the basic principles of soil microbiology and illuminating them with examples from the primary literature accumulated over the short history of this discipline. Any soil microbiology textbook, no matter how complete, can only provide a snapshot of the current status of our studies. A viable scientific endeavor must be continually growing. Great gains in describing the soil environment have been made, but the activities of society have created environmental challenges that may baffle even the most learned soil microbiologist. The challenges of reclaiming severely damaged soil sites, the goals of properly managing our terrestrial home, and the necessity of manipulating the soil system to provide the needs of an ever-growing, ever-changing society demand refinement of our knowledge of the capacity of the soil microbes to adapt to their continually evolving physical home and our capability to manage this minute soil community to meet both their and our needs for survival.

Thus, those that have mastered the introduction to soil microbiology provided within these pages are now challenged to use the information as a form of intellectual energy to advance our knowledge base into the realms necessary to meet the demands of the twenty-first century. As our society entered the more modern era, soil microbiology would have been viewed as a diversion into elucidation of the intriguing "wee beasties" of the soil upon which we walk. Soil microbiology must now play more of an integral role in the advancement and application of ecosystem-sustaining stewardship practices.

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