CHAPTER

16

Microorganisms and Organic Pollutants

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16.1 INTRODUCTION

Since before the beginning of the century, we have dumped enormous amounts of waste products into the environment following the principle of "out of sight, out of mind." Until World War II (WWII), these waste products and their effects went largely unnoticed. However, after WWII, the severity of pollution problems, resulting from careless waste disposal, has steadily increased. Today, more than ever, the general public is aware of these problems.

Historically, sources of waste products have been both industrial and agricultural. For instance, the energy production industry generates huge amounts of waste during the processing of coal and oil and also nuclear energy production. In the past, these wastes have been buried, sometimes not so carefully, and as a result waste constituents have migrated through the soil into groundwater supplies. This type of contamination is said to be from a **point source.** On the other hand, the agricultural sector is the nation's largest user of pesticides and fertilizers. The application of pesticides and fertilizers over vast land areas is responsible for what is called **nonpoint source** contamination. Studies have shown that these agricultural chemicals are being found in our surface water and groundwater supplies in increasing amounts. A 1984 EPA report on nonpoint source pollution identified 44 states where agriculture is an identified nonpoint pollution problem. In 1988, EPA reported that 26 states had confirmed various amounts of 46 different pesticides in their groundwater resulting from normal agricultural practices. Eighteen of these pesticides (in 24 out of the 26 states) were discovered at levels equal to or greater than health advisory levels established or proposed by EPA. Groundwater contamination is a critical issue because groundwater is a major source of potable water. In fact, groundwater constitutes 96% of all available fresh water in the United States (USDA, 1990). Fifty percent of the general population and at least 95% of rural residents obtain drinking water from groundwater sources.

The objective of this chapter is to examine microbial interactions with organic pollutants that can be harnessed to help prevent contamination and clean up contaminated sites. As will be seen in the following section, the United States has passed a series of environmental laws mandating the cleanup of such sites. However, the cost of cleanup has been estimated to be in the trillions of dollars. Therefore, we as a society are reexamining the cleanup issue from several perspectives. The first is related to the cleanup target and the question "How clean is clean?" As you can imagine, the stricter the cleanup provision (e.g., lower contaminant concentration), the greater the attendant cleanup costs. It may require tens to hundreds of millions of dollars for complete cleanup of a large, complex hazardous waste site. In fact, it may be impossible to clean many sites completely. It is very important, therefore, that the physical feasibility of cleanup and the degree of potential risk posed by the contamination be weighed against the economic impact and the future use of the site. The consideration of the future use of the site will help focus our scarce resources on the sites

that pose the greatest current and future risk. The second perspective being considered is whether natural microbial activities in the environment can aid in the cleanup of contaminated sites, and whether these activities will occur rapidly enough naturally, or whether they can and should be enhanced. These two perspectives are closely tied together because although microbial activities can reduce contamination significantly, they often do not remove contamination entirely.

16.2 ENVIRONMENTAL LAW

Society began responding to environmental concerns long ago, beginning with the recognition that our environment is fragile and human activities can have a great impact on it. This led to the creation of Yellowstone National Park in 1872 and the assignment of the care of forested public domain lands to the U.S. Forest Service in 1897. After World War II, as the pollution impacts of industrialization began to be apparent, Congress began to legislate in the area of pollution control. This legislation culminated in the major federal pollution control statutes of the 1970s that now constitute a large body of law called **environmental law.** Federal environmental law consists of laws in the conservation and pollution control areas, along with key planning and coordination statutes. Environmental law is constantly changing and evolving as we try to respond to shifting priorities and pressures on resources. Sometimes changes are made to the law to allow further contamination or risk of contamination to occur for the "good of society." An example is oil exploration in Alaska and off the coast of California. On the other hand, some laws can be made more stringent. Again, using an example from the oil industry, whereas refinery wastes are heavily regulated for disposal, the same types of wastes generated in an oil field were not regulated and were routinely buried without treatment. When attention was drawn to this practice, laws were enacted to require the oil industry to implement proper oil field disposal practices. As these examples suggest, environmental law comprises a complex body of laws, regulations, and decisions now established in the United States. This body of law, which evolved quite quickly in comparison with labor, tax, banking, and communications laws, already ranks with these other areas in size and complexity (Arbuckle *et al.*, 1987).

The term "environmental law" came into being with the enactment of the **National Environmental Policy Act (NEPA)** in early 1970. Although environmental law can vary considerably from state to state and even from city to city, a series of major federal environmental protection laws have been enacted that pertain to the generation, use, and disposition of hazardous waste. NEPA requires each agency, government, or industry that proposes a major action which may have a significant effect on the human environment to prepare an **environmental impact statement (EIS).** The EIS must address the environmental impact of the proposed action and any reasonable alternatives that may exist. The types of projects that NEPA covers are landfills, roads, dams, building complexes, research projects, and any private endeavor requiring a federal license that may affect the environment. *NEPA does not mandate particular results and it does not require a federal agency to adopt the least environmentally damaging alternative.* Because of this, NEPA can be thought of as an "environmental full disclosure law," which requires the applicant to take a "hard look" at the environmental consequences of its action. Thus, an EIS allows environmental concerns and planning to be integrated into the early stages of project planning. Unfortunately, an EIS is often done as an afterthought and becomes a rationale for a project that may be a poor alternative.

Table 16.1 shows a series of environmental laws that have been passed since NEPA to protect our natural resources. These include laws such as the **Clean Air Act** and the **Clean Water Act** that protect air and water resources. There are also laws that govern the permitting of the sale of hazardous chemicals and laws that mandate specific action to be taken in the cleanup of contaminated sites. When the **Comprehensive Environmental Response, Compensation and Liability Act (CERCLA),** more commonly known as **Superfund,** was enacted, it became clear that technology for cleaning up hazardous waste sites was needed. Early remedial actions for contaminated sites consisted of excavation and removal of the contaminated soil to a landfill. Very soon, it became apparant that this was simply moving the problem around, not solving it. As a result, the **Superfund Amendments and Reauthorization Act (SARA)** was passed in 1986. This act added several new dimensions to CERCLA. SARA stipulates cleanup standards and mandates the use of the **National Contingency Plan** to determine the most appropriate action to take in site cleanup.

Two types of responses are available within Superfund: (1) removal actions in response to immediate threats, e.g., removing leaking drums, and (2) remedial actions, which involve cleanup of hazardous sites. The Superfund provisions can be used when a hazardous substance is released or there is a substantial threat of a release that poses imminent and substantial endanger-

TABLE 16.1 History of Environmental Law

Law	Year passed	Goals
Clean Air Act (CAA)	1970	Sets nationwide ambient air quality standards for conventional air pollutants. Sets standards for emissions from both stationary and mobile sources (e.g., motor vehicles).
Clean Water Act (CWA)	1972	Mandates "fishable/swimmable" waters wherever attainable. Provides for (1) a construction grants program for publicly owned water treatment plants and requires plants to achieve the equivalent of secondary treatment; (2) a permit system to regulate point sources of pollution; (3) areawide water quality management to reduce nonpoint sources of pollution; (4) wetlands protection, sludge disposal and ocean discharges; (5) regulation of cleanup of oil spills.
Surface Mining Control and Reclamation Act (SMCRA)	1977	Regulates coal surface mining on private lands and strip mining on public lands. Prohibits surface mining in environmentally sensitive areas.
Resource Conservation and Recovery Act (RCRA)	1976	Provides a comprehensive management scheme for hazardous waste disposal. This includes a system to track the transportation of wastes and federal performance standards for hazardous waste treatment, storage, and disposal facilities. Open dumps are prohibited.
Toxic Substances Control Act (TOSCA)	1976	Requires premarket notification of EPA by the manufacturer of a new chemical. Based on testing information submitted by the manufacturer or premarket test ordered by EPA (including biodegradability and toxicity), a court injunction can be obtained barring the chemical from distribution or sale. EPA can also seek a recall of chemicals already on the market. It is this act that prohibits all but closed-circuit uses of PCBs.
Comprehensive Environmental Response, Compensation and Liability Act (CERCLA)	1980	Commonly known as Superfund, this act covers the cleanup of hazardous substance spills, from vessels, active, or inactive facilities. Establishes a Hazardous Substances Response Trust Fund, financed by a tax on the sale of hazardous chemicals, to be used for removal and cleanup of hazardous waste releases. Cleanup costs must be shared by the affected state. Within certain limits and subject to a few defenses, anyone associated with the release is strictly liable to reimburse the fund for cleanup costs, including damage to natural resources.
Superfund Amendments and Reauthorization Act (SARA)	1986	SARA provides cleanup standards and stipulates rules through the National Contingency Plan for the selection and review of remedial actions. It strongly recommends that remedial actions use on-site treatments that "permanently and significantly reduce the volume, toxicity, or mobility of hazardous substances" and requires remedial action that is "protective of human health and the environment, that is cost-effective, and that utilizes permanent solutions and alternative treatment technologies or resource recovery technologies to the maximum extent practicable."
National Contingency Plan (NCP)	1988	A five-step process to use in evaluation of contaminated sites and suggest the best plan for remediation.

ment to public health and welfare. The process by which Superfund is applied to a site is illustrated in Fig. 16.1. The first step is to place the potential site in the **Superfund Site Inventory.** After a preliminary assessment and site inspection, the decision is made as to whether or not the site will be placed on the **National Priority List (NPL).** Sites placed on this list are those deemed to require a remedial action. Currently, there are more than 1200 sites on the NPL. **The National Contingency Plan (NCP)** is the next component. The purpose of the NCP is to characterize the nature and extent of risk posed by contamination and to evaluate potential remedial options. The investigation and feasibility study components are normally conducted concurrently and with a "phased" approach. This allows feedback between the two components. A diagram of the NCP procedure is provided in Fig. 16.2. The selection of the specific remedial action to be used at a particular site is a very complex process. The goals of the remedial action are that it be protective of human health and the environment, that it maintain protection over time, and that it maximize waste treatment.

16.3 THE OVERALL PROCESS OF BIODEGRADATION

The remainder of this chapter deals with biodegradation of organic contaminants and ways in which these processes can be harnessed to remediate contam-

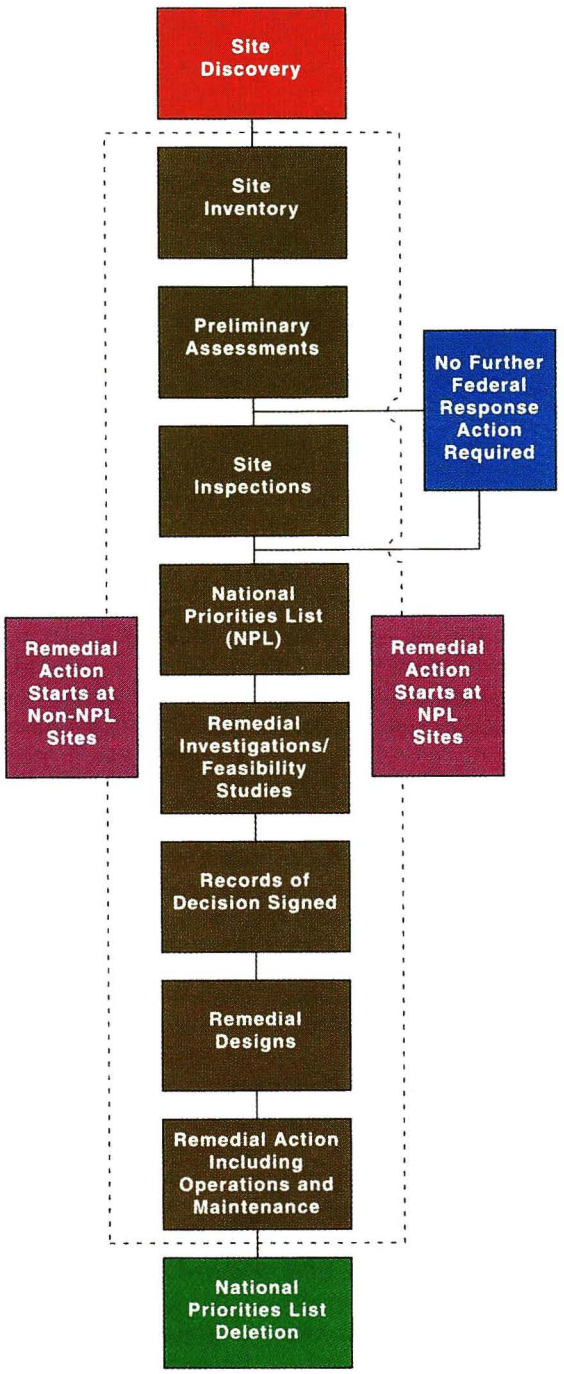


FIGURE 16.1 The Superfund process for treatment of a hazardous waste site. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)

inated sites. **Biodegradation** is the breakdown of organic contaminants that occurs due to microbial activity. As such, these contaminants can be considered as the microbial food source or **substrate.** Biodegradation of any organic compound can be thought of as a series of biological degradation steps or a pathway that ultimately results in the oxidation of the parent compound. Often, the degradation of these compounds results in the generation of energy as described in Chapter 3.

Complete biodegradation or **mineralization** involves oxidation of the parent compound to form carbon dioxide and water, a process that provides both carbon and energy for growth and reproduction of cells. Figure 16.3 illustrates the mineralization of an organic compound under either aerobic or anaerobic conditions. The series of degradation steps constituting mineralization is similar whether the carbon source is a simple sugar such as glucose, a plant polymer such as cellulose, or a pollutant molecule. Each degradation step in the pathway is catalyzed by a specific **enzyme** made by the degrading cell. Enzymes are most often found within a cell but are also made and released from the cell to help initiate degradation reactions. Enzymes found external to the cell are known as **extracellular enzymes.** Extracellular enzymes are important in the degradation of macromolecules such as the plant polymer cellulose (see Chapter 14.2.4.1). Macromolecules must be broken down into smaller subunits outside the cell to allow transport of the smaller subunits into the cell. Degradation by either internal or extracellular enzymes will stop at any step if the appropriate enzyme is not present (Fig. 16.4). Lack of appropriate biodegrading enzymes is one common reason for persistence of organic contaminants, particularly those with unusual chemical structures that existing enzymes do not recognize. Thus, contaminant compounds that have structures similar to those of natural substrates are normally easily degraded. Those that are quite dissimilar to natural substrates are often degraded slowly or not at all.

Some organic contaminants are degraded partially but not completely. This can result from absence of the appropriate degrading enzyme as mentioned earlier. A second type of incomplete degradation is **cometabolism,** in which a partial oxidation of the substrate occurs but the energy derived from the oxidation is not used to support microbial growth. The process occurs when organisms coincidentally possess enzymes that can degrade a particular contaminant. Thus, such enzymes are nonspecific. Cometabolism can occur during periods of active growth or can result from interaction of resting (nongrowing) cells with an organic compound. Cometabolism is difficult to measure in the environment but has been demonstrated for some environmental contaminants. For example, the industrial solvent trichloroethylene (TCE) can be oxidized cometabolically by methanotrophic bacteria that grow on methane as a sole carbon source. TCE is currently of great interest for several reasons. It is one of the most frequently reported contaminants at hazardous waste sites, it is a suspected carcinogen, and it is generally re-

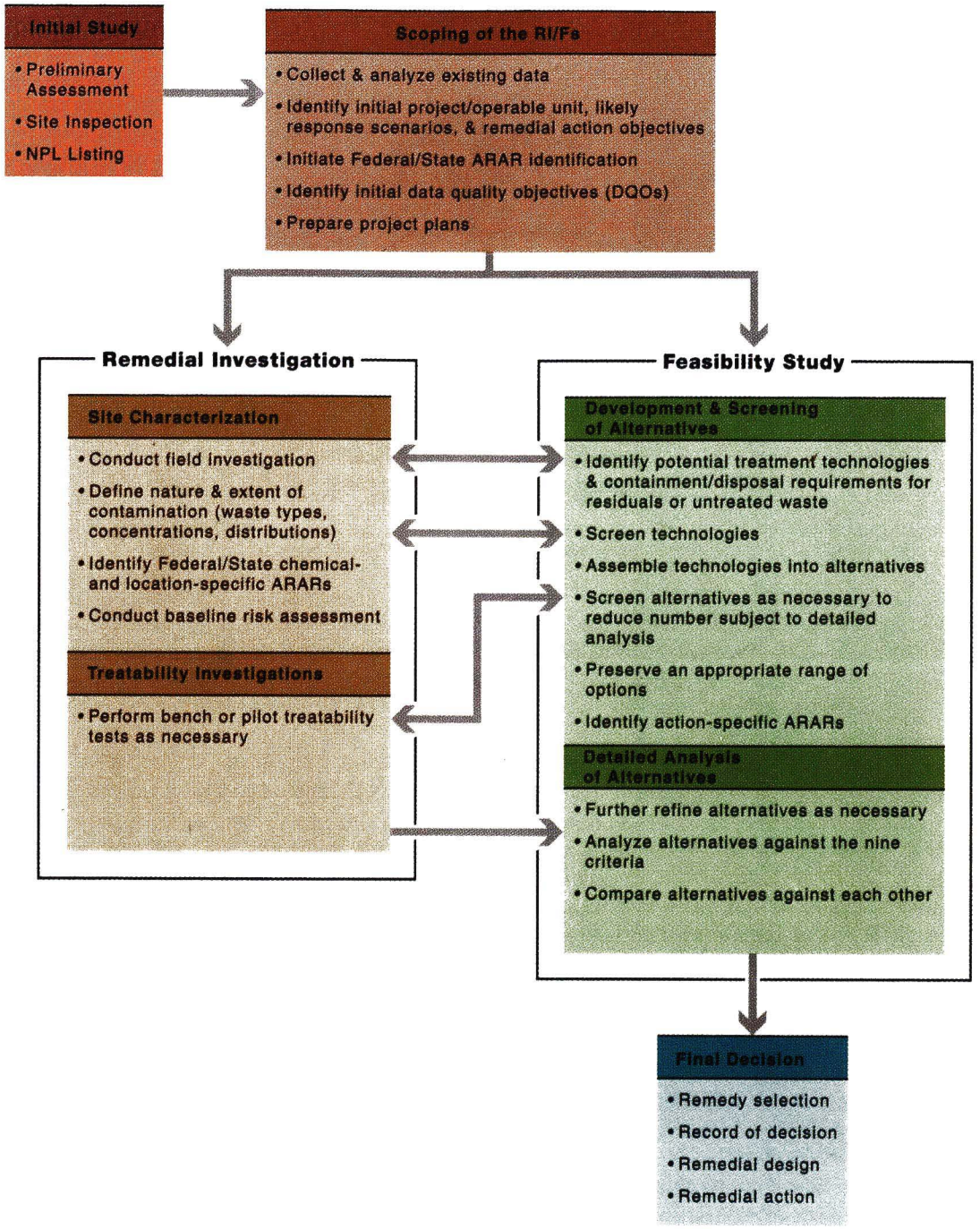


FIGURE 16.2 The remedial investigation–feasibility study (RI/FS) process. ARAR(s) = Applicable or relevant and appropriate requirements. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)

sistant to biodegradation. As shown in Fig. 16.5, the first step in the oxidation of methane by **methanotrophic bacteria** is catalyzed by the enzyme **methane monooxygenase.** This enzyme is so nonspecific that it can also cometabolically catalyze the first step in the oxidation of TCE when both methane and TCE are present. The bacteria receive no energy benefit from this cometabolic degradation step. The subsequent degradation steps shown in Figure 16.5 may be catalyzed spontaneously, by other bacteria, or in some cases by the methanotroph. This is an example of a cometabolic reaction that may have great significance in remediation. Research is currently investigating the application of these methanotrophs to TCE-contaminated sites. Other cometaboliz-

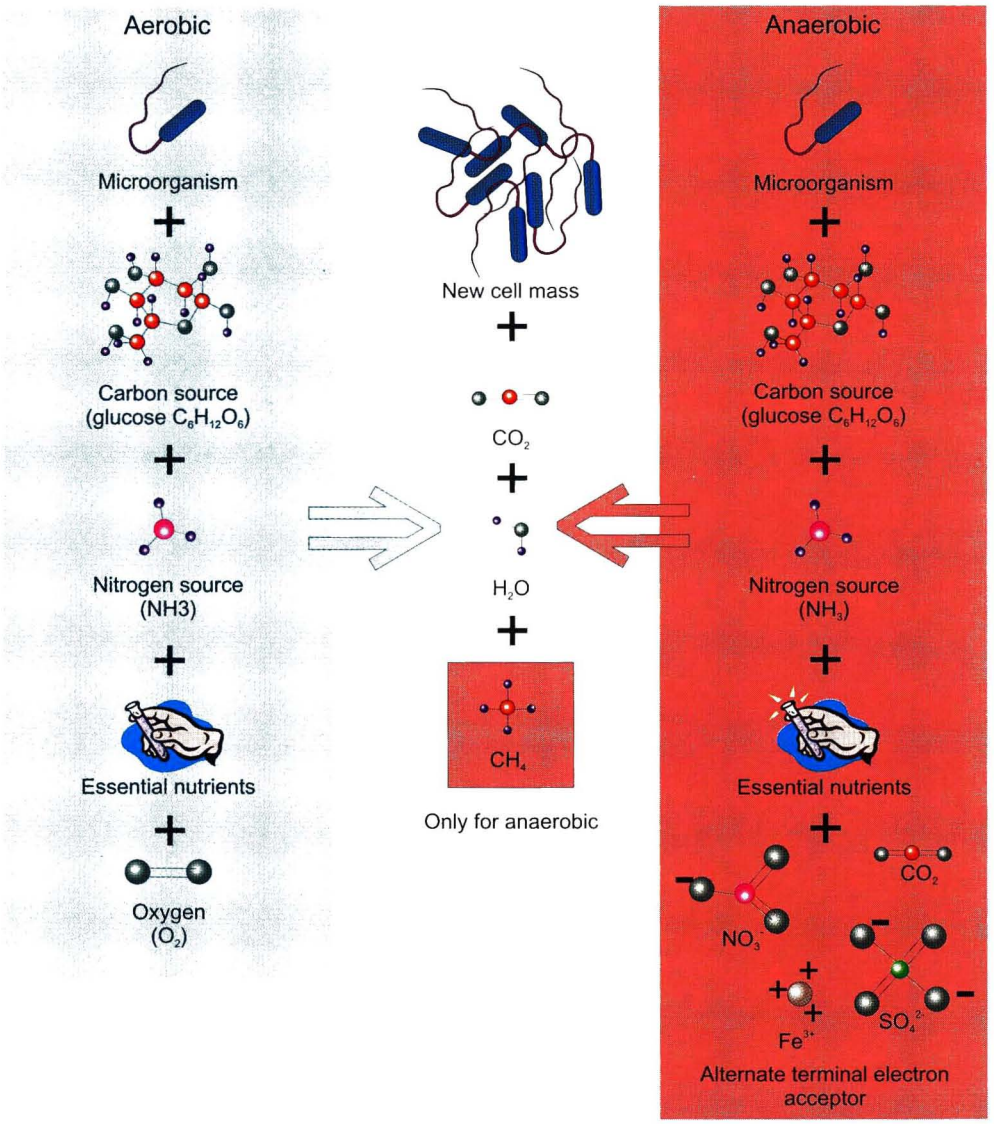


FIGURE 16.3 Aerobic (blue) or anaerobic (red) mineralization of an organic compound.

ing microorganisms that grow on toluene, propane, and even ammonia are also being evaluated for use in TCE bioremediation.

Partial or incomplete degradation can also result in **polymerization** or synthesis of compounds more complex and stable than the parent compound. This occurs when initial degradation steps, often catalyzed by extracellular enzymes, create reactive intermediate compounds. These highly reactive intermediate compounds can then combine with each other or with other organic matter present in the environment. This is illustrated in Fig. 16.6, which shows some possible polymerization reactions that occur with the herbicide propanil during biodegradation. These include formation of dimers or larger polymers, which are quite stable in the environment. Stability is due to low bioavailability (high sorption and low solubility), lack of degrading enzymes, and the fact that some of these residues become chemically bound to the soil organic matter fraction.

16.4 RELATIONSHIP BETWEEN CONTAMINANT STRUCTURE, TOXICITY, AND BIODEGRADABILITY

The vast majority of the organic carbon available to microorganisms in the environment is material that has been photosynthetically fixed (plant material). Of concern are environments that receive large additional inputs of carbon from agriculture or industry (petroleum products, organic solvents, pesticides).

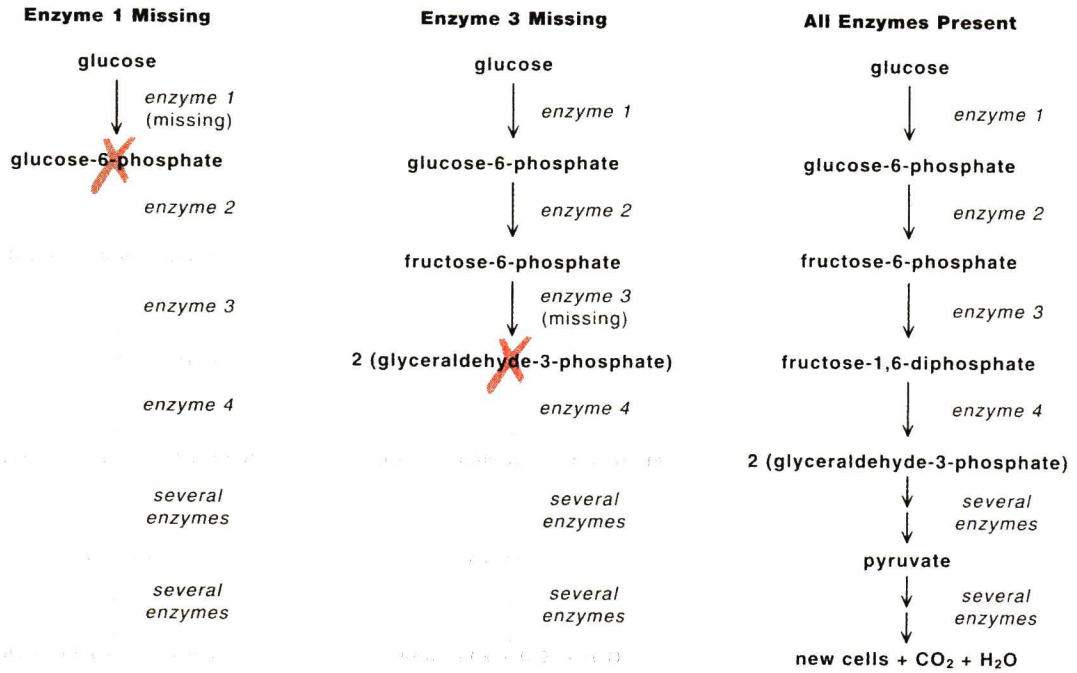


FIGURE 16.4 Stepwise degradation of organic compounds. A different enzyme catalyzes each step of the biodegradation pathway. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)

Although many of these chemicals can be readily degraded because of their structural similarity to naturally occurring organic carbon, the amounts added may exceed the existing **carrying capacity** of the environment. Carrying capacity is defined here as the maximum level of microbial activity that can be expected under existing environmental conditions. Microbial activity may be limited by both biological and physical–chemical factors. These factors include low numbers of microbes, insufficient oxygen or nutrient availability, as well as suboptimal temperature and water availability. These factors are discussed further in Section 16.5. Microbial activity, whether degradation occurs and the rate of degradation, also depends on several factors related to the structure and physical–chemical properties of the contaminant (Miller and Herman, 1997). These factors include

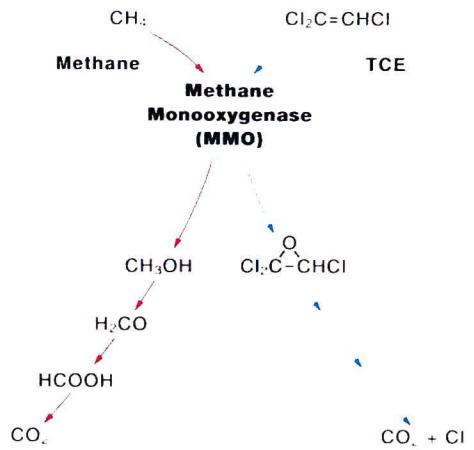


FIGURE 16.5 The oxidation of methane by methanotrophic bacteria is catalyzed by the enzyme methane monooxygenase. Subsequent degradation steps may be catalyzed spontaneously, by other bacteria, or in some cases by the methanotroph. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)

1. Genetic potential, or the presence and expression of appropriate degrading genes by the indigenous microbial community.
2. Bioavailability, or the effect of limited water solubility and sorption on the rate at which a contaminant is taken up by a microbial cell.
3. Contaminant structure, including both steric and electronic effects. Steric effects involve the extent to which substituent groups on a contaminant molecule sterically hinder recognition by the active site of the degrading enzyme. Electronic effects involve the extent to which substituent groups electronically interfere with the interaction between the active site of the enzyme and the contaminant. Electronic effects can also alter the energy required to break critical bonds in the molecule.
4. Toxicity, or the inhibitory effect of the contaminant on cellular metabolism.

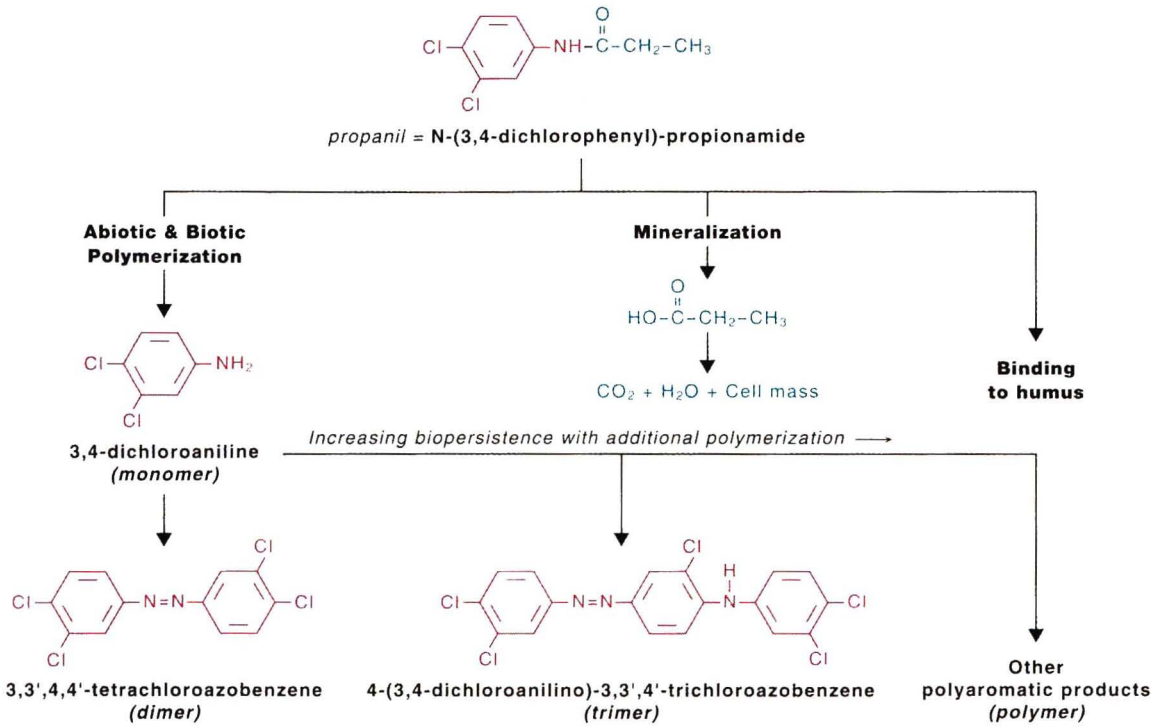


FIGURE 16.6 Polymerization reactions that occur with the herbicide propanil during biodegradation. Propanil is a selective postemergence herbicide used in growing rice. It is toxic to many annual and perennial weeds. The environmental fate of propanil is of concern because it, like many other pesticides, is toxic to most crops except for cereal grains. It is also toxic to fish. Care is used in propanil application to avoid contamination of nearby lakes and streams. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)

16.4.1 Genetic Potential

The onset of contaminant biodegradation generally follows a period of adaptation or acclimation of indigenous microbes, the length of which depends on the contaminant structure. The efficient cycling of plant-based organic matter by soil microorganisms can promote the rapid degradation of organic contaminants that have a chemical structure similar to those of natural soil organic compounds. Previous exposure to a contaminant through repeated pesticide applications or through frequent spills will create an environment in which a biodegradation pathway is maintained within an adapted community. Adaptation of microbial populations most commonly occurs by induction of enzymes necessary for biodegradation followed by an increase in the population of biodegrading organisms (Leahy and Colwell, 1990).

Naturally occurring analogues of certain contaminants may not exist, and previous exposure may not have occurred. Degradation of these contaminants requires a second type of adaptation that involves a genetic change such as a mutation or a gene transfer (Boyle, 1992; Fulthorpe and Wyndham, 1992; Van der Meer *et al.*, 1992). This results in the development of new metabolic capabilities. The time needed for an adaptation requiring a genetic change or for the selection and development of an adapted community is not yet predictable, but it may require weeks to years or may not occur at all.

16.4.2 Bioavailability

For a long time biodegradation was thought to occur if the appropriate microbial enzymes were present. As a result, most research focused on the actual biodegradative process, specifically the isolation and characterization of biodegradative enzymes and genes. There are, however, two steps in the biodegradative process. The first is the uptake of the substrate by the cell, and the second is the metabolism or degradation of the substrate. Assuming the presence of an appropriate metabolic pathway, degradation of a contaminant can proceed rapidly if the contaminant is available in a water-soluble form. However, degradation of contaminants with limited water solubility or that are strongly sorbed to soil or sediments can be limited due to their low bioavailability (Miller, 1995).

Growth on an organic compound with limited water solubility poses a unique problem for microorganisms because the compound is not freely available in the aqueous phase. Most microorganisms require high

water activity (>0.96) for active metabolism (Atlas and Bartha, 1993), and thus the contact between the degrading organism and an organic compound with low water solubility is limited. The compound may be present in a liquid or solid state, both of which can form a two-phase system with water. Liquid hydrocarbons can be less or more dense than water, forming a separate phase above or below the water surface. For example, polychlorinated biphenyls (PCBs) and chlorinated solvents such as TCE are denser than water and form a separate phase below the water surface. Solvents less dense than water, such as benzene and other petroleum constituents, form a separate phase above the water surface. There are three possible modes of microbial uptake of a liquid organic (Fig. 16.7):

1. Utilization of the solubilized organic compound (Fig. 16.7A).
2. Direct contact of cells with the organic compound. This can be mediated by cell modifications such as fimbriae (Rosenberg *et al.*, 1982) or cell surface hydrophobicity (Zhang and Miller, 1994), which increase attachment of the cell to the organic compound (Fig. 16.7B).
3. Direct contact with fine or submicrometer size substrate droplets dispersed in the aqueous phase (Fig. 16.7C).

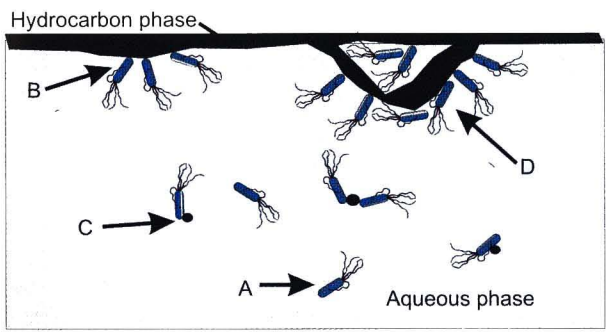


FIGURE 16.7 A water environment with a oil phase floating on the surface. This is typical of what might occur when oil is spilled in the ocean. There are several ways in which microbes reach the oil phase in this type of situation. (A) Microbes can take up hydrocarbons dissolved in the aqueous phase surrounding degrading cells. This uptake mode becomes limiting as the aqueous solubility of the hydrocarbon decreases. (B) Uptake via direct contact of degrading cells at the aqueous–hydrocarbon interface of large oil drops in water. This uptake mode is limited by the interfacial area between the water and hydrocarbon phase. (C) Uptake through direct contact of degrading cells with fine or submicrometer-size oil droplets dispersed in the aqueous phase. This uptake mode is limited by the formation of such droplets. In the ocean environment, wave action can create substantial dispersion of oil. In a soil environment such dispersion is more limited. (D) Enhanced uptake as a result of production of biosurfactants or emulsifiers that effectively increase the apparent aqueous solubility of the hydrocarbon or allow better attachment of cells to the hydrocarbon.

The mode that predominates depends largely on the water solubility of the organic compound. In general, direct contact with the organic compound plays a more important role (modes 2 and 3) as water solubility decreases.

Some microbes can enhance the rate of uptake and biodegradation as a result of production of **biosurfactants** or emulsifiers (Fig. 16.7D). There are two effects of biosurfactants. First, they can effectively increase the aqueous solubility of the hydrocarbon through formation of micelles or vesicles (Fig. 16.8) that associate with hydrocarbons. Second, they can facilitate attachment of cells to the hydrocarbon by making the cell surface more hydrophobic and, thus, better able to stick to a separate oil phase (Fig. 16.9). This makes it possible to achieve greatly enhanced biodegradation rates in the presence of biosurfactants (Herman *et al.*, 1997).

For organic compounds in the solid phase, e.g., waxes, plastics, or polyaromatic hydrocarbons (PAHs), there are only two modes by which a cell can take up the substrate:

1. Direct contact with the substrate
2. Utilization of solubilized substrate

Available evidence suggests that for solid-phase organic compounds, utilization of solubilized substrate is most important. Thus, low water solubility has a greater impact on degradation of solid- phase organic compounds than on liquid-phase organics.

Another factor that affects bioavailability of an organic compound is sorption of the compound by soil or sediment (Novak *et al.*, 1995). Depending on the sorption mechanism, organic compounds can be weakly (hydrogen bonding, van der Waals forces, hydrophobic interactions) or strongly (covalent binding) bound to soil. Sorption of weakly bound or labile residues is reversible, and when a sorbed residue is released back into solution it becomes available for microbial utilization (Scow, 1993). Bioavailability can also be reduced by the diffusion of contaminants into soil matrix microsites that are inaccessible to bacteria because of pore size exclusion (Alexander, 1995). There is evidence that the proportion of labile residues made available by desorption decreases with the length of time the residues are in the soil. Thus, as contaminants age and become sequestered more deeply within inaccessible microsites (Fig. 4.3), bioavailability, and therefore biodegradation, can be expected to decrease.

Finally, some contaminants may be incorporated into soil organic matter by the catalytic activity of a wide variety of oxidative enzymes that are present in the soil matrix. The incorporation of contaminants into

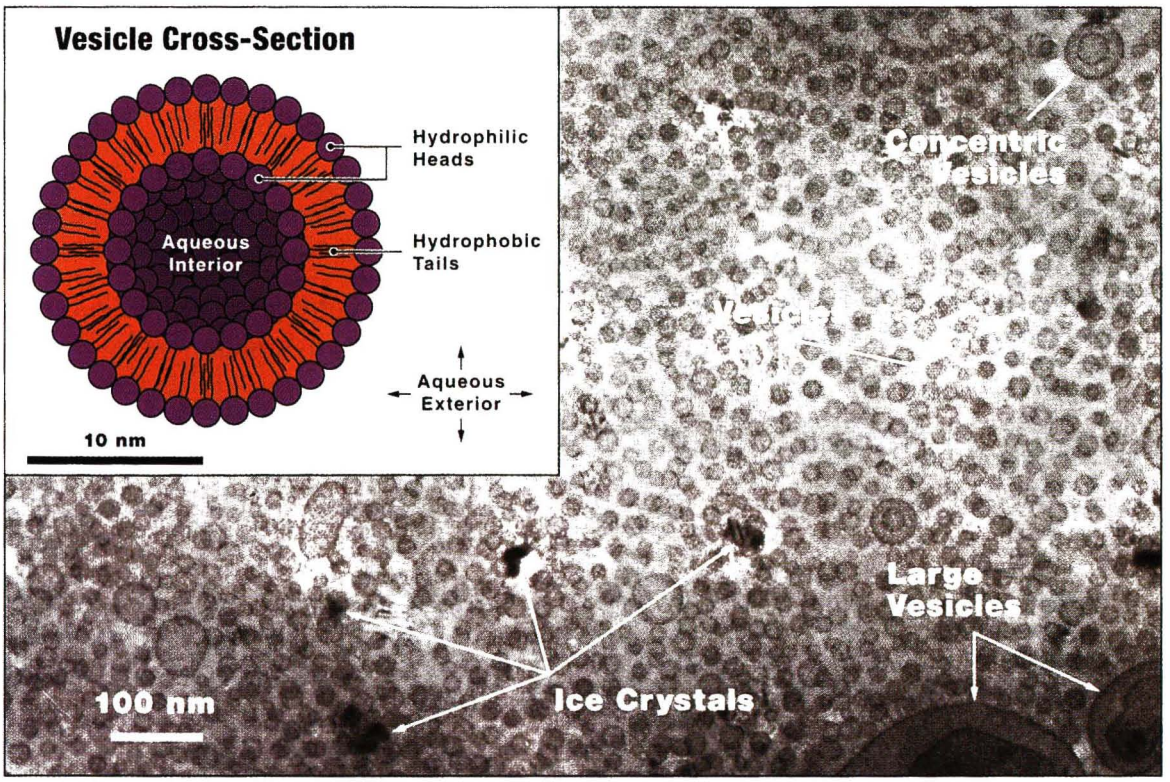


FIGURE 16.8 Cryo–transmission electron micrograph of a microbially produced surfactant, rhamnolipid. In water, this compound spontaneously forms aggregates such as the vesicles shown here. Hydrocarbons like to associate with the lipid-like layer formed by the hydrophobic tails of the surfactant vesicles. These tiny surfactant–hydrocarbon structures are soluble in the aqueous phase. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)

soil organic matter is called **humification,** a process that is usually irreversible and that may be considered as one factor in the aging process (Bollag, 1992). These bound or humified residues are released and degraded only very slowly as part of the normal turnover of humic material in soil (see Chapter 14.2.4.2).

16.4.3 Contaminant Structure

16.4.3.1 Steric Effects

What type of contaminant structures can lead to low degradation rates even if the contaminant structure is similar to naturally-occurring molecules? It is the presence of branching and functional groups that often slows degradation by changing the chemistry of the degradation **reaction site.** The reaction site is where a degradative enzyme comes into contact with a contaminant substrate causing a transformation step to occur. When the reaction site is blocked by branching or a functional group, contact between the contaminant and enzyme at the reaction site is hindered. This is known as a steric effect and is illustrated in Fig. 16.10, which compares two structures, an eight-carbon *n*-alkane (A) and the same eight-carbon backbone with four methyl branches (B). Whereas octane is readily degradable by the pathway shown in Fig. 16.14, the four methyl substituents in structure B inhibit degradation at both ends of the molecule. Branching or functional groups can also affect transport of the substrate across the cell membrane, especially if the transport is enzyme-assisted. Steric effects usually increase as the size of the functional group increases (Pitter and Chudoba, 1990).

16.4.3.2 Electronic Effects

In addition to steric effects, functional groups may contribute electronic effects that hinder biodegradation by affecting the interaction between the contaminant and the enzyme. Functional groups can be electron-donating (e.g., CH_3) or electron-withdrawing (e.g., Cl), and therefore, can change the electron density of the reaction site. In general, functional groups that add to the electron density of the reaction site increase biodegradation rates, and functional groups that decrease the electron density of the reaction site decrease biodegradation rates. To illustrate the relation-

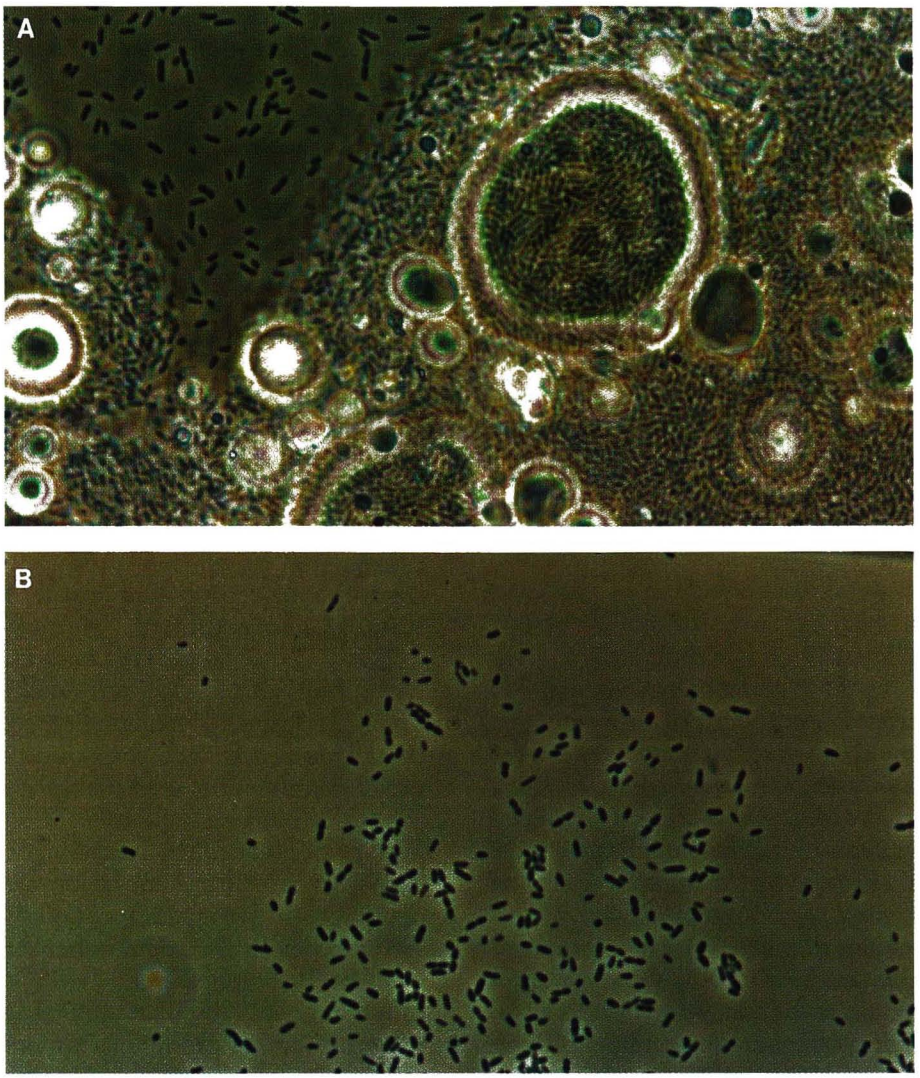


FIGURE 16.9 Phase-contrast micrographs showing the effect of a biosurfactant on the ability of *Pseudomonas aeruginosa* ATCC 15524 to stick to hexadecane droplets (magnification × 1000). (A) Addition of rhamnolipid biosurfactant (0.1 mM) causes cells to clump and to stick to oil droplets. (B) No biosurfactant is present and individual cells do not clump and do not stick to oil droplets in the solution. (Photos courtesy D. C. Herman.)

ship between functional group electronegativities and rate of biodegradation, Pitter and Chudoba (1990) compared the electronegativity of a series of ortho-substituted phenols with their biodegradation rates. Five different functional groups were tested, and it was found that as the electronegativity of the substituents increased, biodegradation rates decreased (Fig. 16.11).

16.4.4 Toxicity

Contaminant spills and engineered remediation projects, such as landfarming of petroleum refinery sludges, can involve extremely high contaminant concentrations. In these cases, toxicity of the contaminant to microbial populations can slow the remediation process. The toxicity of nonionized organic contaminants such as petroleum hydrocarbons or organic solvents is to a large extent due to a nonspecific narcotic-type mode of action. Nonspecific narcotic-type toxicity is based on the partitioning of a dissolved contaminant into the lipophilic layer of the cell membrane, which causes a disruption of membrane integrity (Sikkema *et al.*, 1995). The cell membrane is believed to be the major site of organic contaminant accumulation in mi-

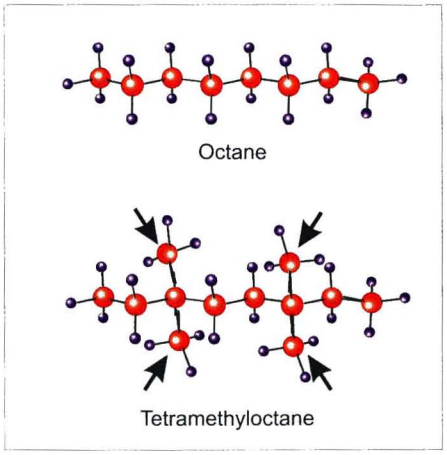


FIGURE 16.10 The structure of (A) octane, which is readily degradable, and (B) a tetramethyl-substituted octane that is not degraded because the methyl groups block the enzyme–substrate catalysis site.

croorganisms. One result of membrane exposure to contaminants is increased permeability of the membrane. The disruption of membrane permeability has been assayed in several ways: using fluorescent dyes (Herman *et al.*, 1991); by the loss of intracellular components, such as potassium from algae (Hutchinson *et al.*, 1981) or bacteria (Bernheim, 1974); or by the loss of ^{14}C-labeled photosynthates from algal cells (Stratton, 1989).

Models have been developed that relate bioconcentration (the accumulation of a hydrophobic contaminant by a cell or organism) and toxicity to physicochemical attributes of the organic contaminant. These models are referred to as **quantitative structure–activity relationship (QSAR)** models. QSAR models allow physical descriptors to be used to predict the toxicity of a wide variety of nonionized organics to aquatic organisms and have also been used to predict the inhibition of microbial activity (Blum and Speece, 1990). Such descriptors include hydrophobicity, as determined from the octanol–water partition coefficient (K_{ow}), and molecular connectivity, which represents the surface topography of a compound. An example of a QSAR model was provided by Warne *et al.* (1989a) who found a strong correlation between growth inhibition of a mixed culture of marine bacteria and the K_{ow} of shale oil components, including alkyl-substituted benzenes, naphthalenes, pyridines, and phenols. Similar correlations were found for the toxicity of mono-, di-, tri-, tetra-, and pentachlorophenols to bacterial growth (Liu *et al.*, 1982). In this case, increasing chlorination of phenols produced a more lipophilic (hydrophobic) compound (greater K_{ow}) that was more toxic to bacterial growth.

Many QSAR models are based on the hydrophobic partitioning of a nonionized organic compound, leading to a nonspecific, narcotic-type mode of action. Exceptions can occur if a compound has a more specific mode of inhibition. For example, steric or electronic factors can increase the toxicity of a compound over that attributed to hydrophobicity alone. This was demonstrated in a comparison of isomers of mono-, di-, tri-, and tetrachlorophenols (Ruckdeschel *et al.*, 1987) and of di- and trichlorobenzenes (Liu and Thomson, 1983). Some isomers with similar K_{ow} values differed greatly in their ability to inhibit microbial growth. Another example is aromatic compounds that can be microbially transformed to highly reactive pheno-

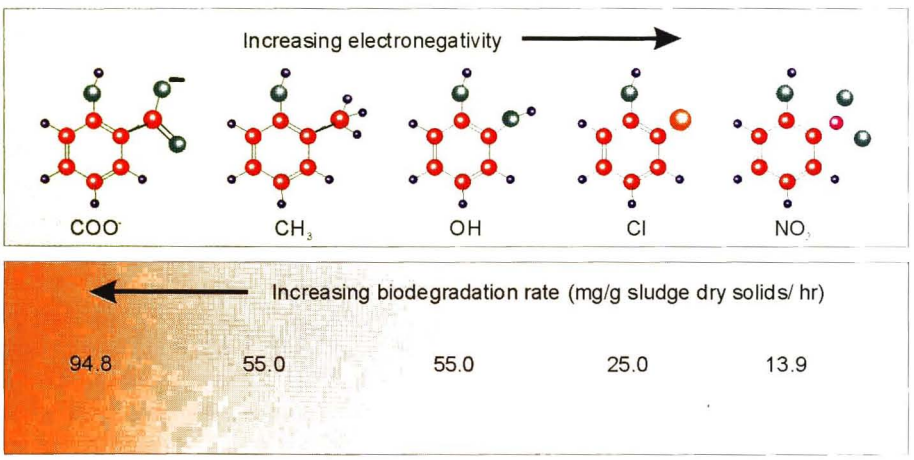


FIGURE 16.11 Various ortho-substituted phenols and their respective biodegradation rates. (Adapted from Pitter and Chudoba, 1990.)

lic or quinone derivatives that have greater toxicity than would be predicted on the basis of hydrophobic partitioning alone (Narro *et al.*, 1992).

One limitation of hydrophobicity-based QSAR modeling is the condition that the organic contaminant is partitioned from an aqueous (solubilized) phase into a lipid phase. This necessitates that a toxic aqueous concentration is achievable within the water solubility limits of a particular compound. Thus, certain organic contaminants, such as high-molecular-weight aliphatic or asphaltene components of crude oil, that are strongly hydrophobic are not thought to be toxic to microorganisms. In contrast, low-molecular-weight aliphatic and aromatic components with higher water solubilities are strongly associated with the toxicity of crude oil to microorganisms.

16.5 ENVIRONMENTAL FACTORS AFFECTING BIODEGRADATION

A number of parameters influence the survival and activity of microorganisms in any environment. One factor that has great influence on microbial activity is organic matter, the primary source of carbon for heterotrophic microorganisms in most environments. Surface soils have a relatively high and variable organic matter content and therefore are characterized by high microbial numbers and diverse metabolic activity (see Chapter 4). In contrast, the subsurface unsaturated (vadose) zone and saturated zone usually have a much lower content and diversity of organic matter, resulting in lower microbial numbers and activity. Exceptions to this rule are some areas of the saturated zone that have high flow or recharge rates, which can lead to numbers and activities of microorganisms similar to those found in surface soils.

Occurrence and abundance of microorganisms in an environment are determined not only by available carbon but also by various physical and chemical factors. These include oxygen availability, nutrient availability, temperature, pH, salinity, and water activity. Inhibition of biodegradation can be caused by a limitation imposed by any one of these factors, but the cause of the persistence of a contaminant is sometimes difficult to determine. Perhaps the most important factors controlling contaminant biodegradation in the environment are oxygen availability, organic matter content, nitrogen availability, and contaminant bioavailability. Interestingly, the first three of these factors can change considerably depending on the location of the contaminant. Figure 16.12 shows the relationship between organic carbon, oxygen, and microbial activity in a profile of the terrestrial ecosystem including surface soils, the vadose zone, and the saturated zone.

16.5.1 Oxygen

Oxygen is very important in determining the extent and rate of contaminant biodegradation. In general, aerobic biodegradation is much faster than anaerobic biodegradation. For example, petroleum-based hydrocarbons entering the aerobic zones of freshwater lakes and rivers are generally susceptible to microbial degradation, but oil accumulated in anaerobic sediments can be highly persistent (Cooney, 1984). It follows that as oxygen is depleted, a reduction in the rate of hydrocarbon degradation can be expected. Oxygen is especially important for degradation of highly reduced hydrocarbons such as the alkane hexadecane ($C_{16}H_{34}$). Biodegradation of hexadecane was found to occur only in the presence of oxygen, although an oxygen tension as low as 1% of full oxygen saturation was enough to allow degradation to occur (Michaelsen *et al.*, 1992).

Benzene, oxygenated aromatics such as benzoate or phenols, and alkylated aromatics such as toluene have been shown to be biodegraded under anaerobic conditions when nitrate, iron, and sulfate are available for use as terminal electron acceptors (Evans and Fuchs, 1988). Nevertheless, it is still accepted that the biodegradation of such compounds is much slower under anaerobic conditions and requires much longer adaptation periods than degradation in an aerobic environment. Some studies have shown that anaerobic biodegradation by specifically adapted consortia can achieve rapid loss of contaminants (Lovley *et al.*, 1995), indicating that anaerobic degradation may become more important as a bioremediation tool as more is understood about this process.

16.5.2 Organic Matter Content

Surface soils have large numbers of microorganisms. Bacterial numbers are generally 10^6 to 10^9 organisms per gram of soil. Fungal numbers are somewhat lower, 10^4 to 10^6 per gram of soil. In contrast, microbial populations in deeper regions such as the deep vadose zone and groundwater region are often lower by two orders of magnitude or more (see Chapter 4). This large decrease in microbial numbers with depth is due primarily to differences in organic matter content. Both the vadose zone and the groundwater region have low amounts of organic matter. One result of low total numbers of microorganisms is that a low popula-

tion of contaminant degraders may be present initially. Thus, biodegradation of a particular contaminant may be slow until a sufficient biodegrading population has been built up. A second reason for slow biodegradation in the vadose zone and groundwater region is that because a low amount of organic matter is present, the organisms in this region are often dormant. This can cause their response to an added carbon source to be slow, especially if the carbon source is a contaminant molecule that has low bioavailability or to which the organisms have not had prior exposure.

Because of these trends in oxygen availability and organic matter content, several generalizations can be made with respect to surface soils, the vadose zone, and the groundwater region (Fig. 16.12):

1. Biodegradation in surface soils is primarily aerobic and rapid.
2. Biodegradation in the vadose zone is also primarily aerobic, but significant acclimation times may be necessary for significant biodegrading populations to build up.
3. Biodegradation in the deep groundwater region is also initially slow because of low numbers, and can rapidly become anaerobic because of lack of available oxygen. Biodegradation in shallow groundwater regions is initially more rapid because of higher microbial numbers but is similarly slowed by low oxygen availability.

16.5.3 Nitrogen

Microbial utilization of organic contaminants, particularly hydrocarbons composed primarily of carbon and hydrogen, creates a demand for essential nutrients such as nitrogen and phosphorus. Ward and Brock (1976) monitored seasonal variations in hydrocarbon degradation in a temperate lake and found that variations in the available forms of nitrogen and phosphorus limited degradation. Maximum rates of hydrocarbon degradation were evident in early spring, when available nitrogen and phosphorus levels were high, but rapid consumption of these nutrients reduced the rate of hydrocarbon degradation during the summer months. Thus, biodegradation can often be improved simply by the addition of nitrogen fertilizers. This is particularly true in the case of biodegradation of petroleum oil spills, in which nitrogen shortages can be acute. In general, microbes have an average C:N ratio within their biomass of about 5:1 to 10:1 depending on the type of microorganism. Therefore a ratio of approximately 100:10:1 (C:N:P) is often used in such sites. However, in some instances, quite different ratios have been used. For example, Wang and Bartha (1990) found that effective remediation of hydrocarbons in soil required the addition of nitrogen and phosphorus additions to maintain a C:N ratio of 200:1 and a C:P ratio of 1000:1. Why were the C:N and C:P ratios maintained at levels so much higher than the cell C:N and C:P ratios? As discussed in Chapter 14.3.3.2, it is because much of the hydrocarbon carbon that is metabolized is released as carbon dioxide so that much of the carbon is lost from the system. In contrast, almost all of the nitrogen and phosphorus metabolized is incorporated into microbial biomass and thus is conserved in the system.

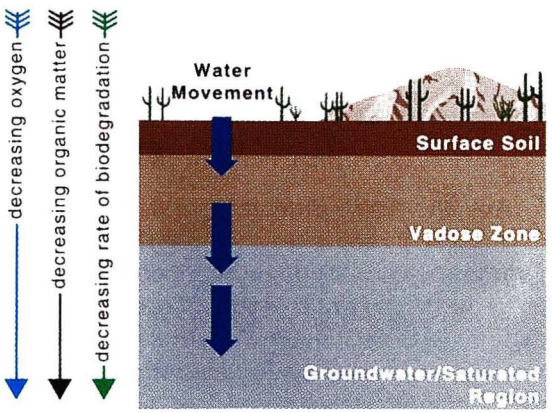


FIGURE 16.12 There are three major locations where contamination can occur in terrestrial ecosystems: surface soils, the vadose zone, and the saturated zone. The availability of both oxygen and organic matter varies considerably in these zones. As indicated, oxygen and organic matter both decrease with depth, resulting in a decrease in biodegradation activity with depth. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)

16.5.4 Other Environmental Factors

16.5.4.1 Temperature

Hydrocarbon degradation has been reported to occur at a range of temperatures between close to freezing and more than 30°C. Bacteria can adapt to temperature extremes in order to maintain metabolic activity; however, seasonal temperature fluctuations in the natural environment have been shown to affect the rate at which degradation occurs (Palmisano *et al.*, 1991). For example, the degradation rates of hexadecane and naphthalene in a river sediment were reduced approximately 4.5-fold and 40-fold, respectively, in winter (0–4°C) compared with summer (8–21°C) samples (Wyndham and Costerton, 1981).

16.5.4.2 pH

In soils, the rate of hydrocarbon degradation is often higher in alkaline conditions than in acidic conditions. In acidic soils, fungi are more competitive than bacteria, which prefer a neutral environment. There-

fore, at lower soil pH, fungi become more important in hydrocarbon degradation. Acidic soils favor the growth of fungi, which degrade hydrocarbons but usually at a slower rate than soil bacteria, which prefer a more neutral to alkaline environment. Hambrick *et al.* (1980) examined the effect of pH on hydrocarbon degradation in a salt marsh sediment. The pH of the sediment ranged from 6.5 to 8.0, but when incubated at different pH levels, lower rates of hydrocarbon degradation were evident at pH 5.0 and 6.5 than at pH 8.0.

16.5.4.3 Salinity

Hydrocarbon degradation has been shown to occur in saline environments. Samples of freshwater sediment incubated under saline conditions showed a reduced rate of hydrocarbon degradation. In contrast, hydrocarbon degradation in estuarine sediments incubated with increasing levels of salinity were little affected (Kerr and Capone, 1988), although hypersaline conditions were reported to reduce the rate of hydrocarbon degradation in sediments sampled from a saline lake (Ward and Brock, 1978).

16.5.4.4 Water Activity

Optimal conditions for activity of aerobic soil microorganisms occur between 38% and 81% of the soil pore space (also referred to as % saturation) because in this range of water contents, water and oxygen availability are maximized. At higher water contents, the slow rate of oxygen diffusion through water limits oxygen replenishment, thereby limiting aerobic activity. At lower water contents, water availability becomes limiting. Why is the optimal % saturation range so broad? It is because optimal activity really depends upon a combination of factors including water content and available pore space. Available pore space is measured as bulk density which is defined as the mass of soil per unit volume (g/cm^3). This means that in any given soil, increasing bulk density indicates increasing compaction of the soil. In a soil that is loosely compacted (lower bulk density), a water saturation of 70% represents more water (more filled small pores and pore throats) than in a highly compacted soil. Thus, for soils of low bulk density, oxygen diffusion constraints become important at lower water saturation than for highly compacted soils.

For example, Neilson and Pepper (1990) showed that respiration in a clay loam soil was maximal at different % saturation depending on bulk density. At a bulk density of 1.1 g/cm^3, respiration was optimal between 38 and 45% water saturation. At a bulk density of 1.6 g/cm^3, respiration was optimal at a higher water saturation, 81%.

16.6 BIODEGRADATION OF ORGANIC POLLUTANTS

16.6.1 Pollutant Sources and Types

In 1981, the United States used almost 6 billion barrels of oil for heating, generation of electricity, and gasoline. Other sources of energy are coal, natural gas, and nuclear energy. The paper, transportation, electronics, defense, and metals industries all produce large amounts of waste, including solvents, acids, bases, and metals. It is estimated that in 1985, 3 million tons ($15.9 billion market value) of pesticides were used worldwide, with the United States using approximately one fourth of this total. As these figures demonstrate, both industry and agriculture produce large amounts of chemicals. Inevitably, some of these find their way into the environment as a result of normal handling procedures and spills. Figure 16.13 shows various contaminant molecules that are added to the environment in significant quantities by anthropogenic activities. The structure of most contaminant molecules is based on the one of the three structures shown in Fig. 16.13: aliphatic, alicyclic, or aromatic. By combining or adding to these structures, a variety of complex molecules can be formed that have unique properties useful in industry and agriculture. The objective of this section is to become familiar with these structures and their biodegradation pathways so that given a structure, a reasonable biodegradation pathway can be predicted.

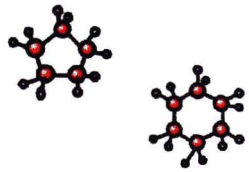
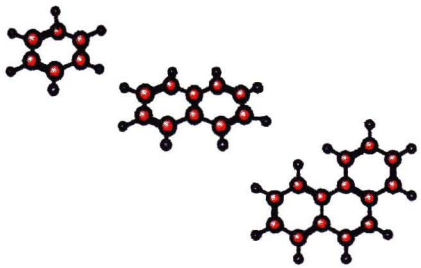
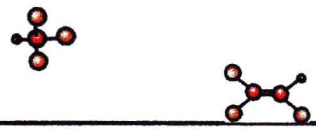
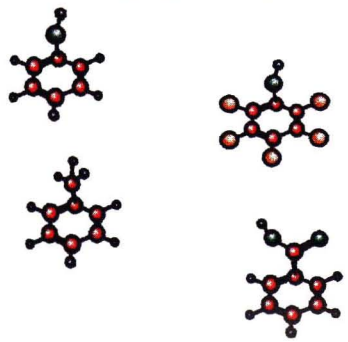
16.6.2 Aerobic Conditions

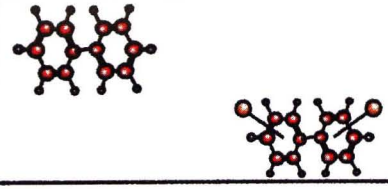
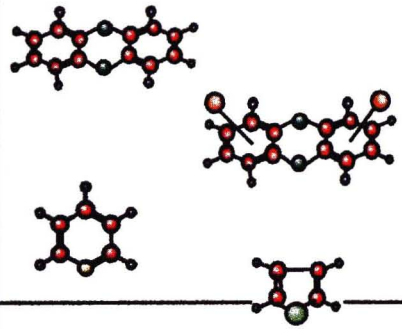
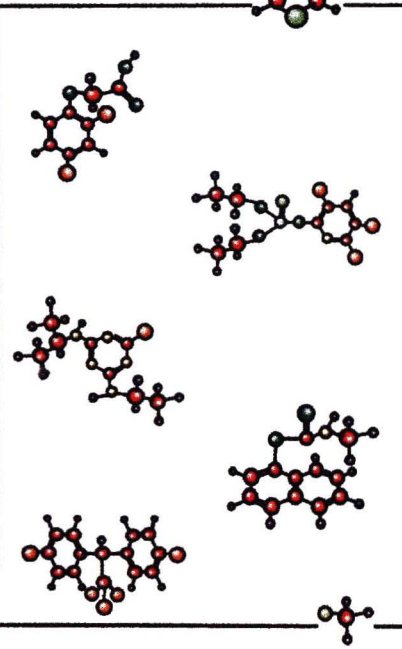
16.6.2.1 Aliphatics

There are several common sources of aliphatic hydrocarbons that enter the environment as contaminants. These include straight-chain and branched-chain structures found in petroleum hydrocarbons, the linear alkyl benzenesulfonate (LAS) detergents, and the one- and two-carbon halogenated compounds such as TCE that are commonly used as industrial solvents. Some general rules for aliphatic biodegradation are presented in Information Box 1, and specific biodegradation pathways are summarized in the following sections for alkanes, alkenes, and chlorinated aliphatics.

Alkanes

Because of their structural similarity to fatty acids and plant paraffins, which are ubiquitous in nature, many microorganisms in the environment can utilize *n*-alkanes (straight-chain alkanes) as a sole source of carbon and energy. In fact, it is easy to isolate alkane-

Hydrocarbon Type	Structure	Name	Physical state at room temp.	Source and uses
Aliphatics		propane n=1 hexane n=4 hexatriacontane n=34	gas liquid solid	Petroleum contains both linear and branched aliphatics. The gasoline fraction of crude oil is 30 - 70% aliphatic depending on the source of the crude oil.
Alicyclics		cyclopentane cyclohexane	liquid liquid	Petroleum contains both unsubstituted and alkyl substituted alicyclics. The gasoline fraction of crude oil is 20-70% alicyclic depending on the source of the crude oil
Aromatics		benzene naphthalene phenanthrene	liquid solid solid	Petroleum contains both unsubstituted and alkyl substituted aromatics. The gasoline fraction of crude oil is 10-15% depending on the source of the crude oil.
Substituted aliphatics		chloroform trichloroethylene (TCE)	liquid liquid	Anthropogenically manufactured and used as solvents, degreasing agents, and in organic syntheses.
Substituted aromatics		phenol pentachlorophenol toluene benzoate	liquid liquid liquid liquid	Found in coal tar or manufactured and used as a disinfectant, and in manufacture of resins, dyes and industrial chemicals. Manufactured and used as an insecticide, defoliant, and wood preservative. Found in tar oil, used in manufacture of organics, explosives, and dyes. Also used as a solvent. Found in plants and animals and manufactured for use as a food preservative, dye component, and in curing tobacco.

Biaryl hydrocarbons		biphenyl	solid	Biphenyl is the parent compound of variously chlorinated biphenyl mixtures known as the PCBs. PCBs are used as transformer oils and plasticizers.
		polychlorinated biphenyls (PCBs)	liquid	
Heterocyclics		dibenzodioxin	solid	Dioxins are created during incineration processes and are contaminants associated with the manufacture of herbicides including 2,4-D and 2,4,5-T.
		chlorinated dioxins	solid	
		pyridine	liquid	Found in coal tar. Used as a solvent and synthetic intermediate.
		thiophene	liquid	Found in coal tar, coal gas and crude oil. Used as a solvent and in manufacture of resins, dyes, and pharmaceuticals.
Pesticides Organic acids		2,4-dichlorophenoxy acetic acid	solid	Broadleaf herbicide
Organophosphates		chlorpyrifos	solid	Used as an insecticide and an acaricide
Triazenes		atrazine	solid	Selective herbicide
Carbamates		carbaryl	solid	Contact insecticide
Chlorinated hydrocarbons		1,1,1-trichloro-2,2-bis-(4-chlorophenyl)-ethane (DDT)	solid	Contact insecticide
		methyl bromide	gas	Used to degrease wool, extract oil from nuts, seeds and flowers, used as an insect and soil fumigant.

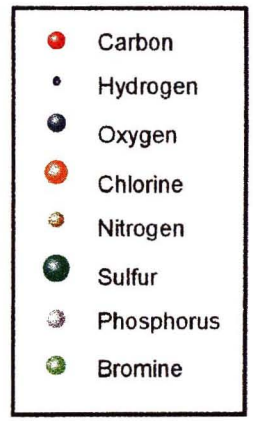


FIGURE 16.13 Representative pollutant structures.

Information Box 1

General rules for degradation of aliphatic compounds:

1. Midsize straight-chain aliphatics (*n*-alkanes C_{10} to C_{18} in length) are utilized more readily than *n*-alkanes with either shorter or longer chains.
2. Saturated aliphatics and alkenes are degraded similarly.
3. Hydrocarbon branching decreases biodegradability.
4. Halogen substitution decreases biodegradability.

degrading microbes from any environmental sample. As a result, alkanes are usually considered to be the most readily biodegradable type of hydrocarbon. Biodegradation of alkanes occurs with a high biological oxygen demand (BOD) using one of the two pathways shown in Fig. 16.14. The more common pathway is the direct incorporation of one atom of oxygen onto one of the end carbons of the alkane by a **monooxygenase enzyme.** This results in the formation of a primary alcohol (see pathway I). Alternatively, a **dioxygenase enzyme** can incorporate both oxygen atoms into the alkane to form a hydroperoxide (pathway II). The end result of both pathways is the production of a primary fatty acid. There are also examples in the literature of diterminal oxidation, with both ends of the alkane oxidized, and of subterminal oxidation, with an interior carbon oxidized (Britton, 1984).

Fatty acids are common metabolites found in all cells. They are used in the synthesis of membrane phospholipids and lipid storage materials. The common pathway used to catabolize fatty acids is known as **β-oxidation,** a pathway that cleaves off consecutive two-carbon fragments (Fig. 16.14). Each two carbon fragment is removed by coenzyme A as acetyl-CoA, which then enters the tricarboxylic acid (TCA) cycle for complete mineralization to CO_2 and H_2O. If you think about this process, it becomes apparent that if one starts with an alkane that has an even number of carbons, the two-carbon fragment acetyl-CoA will be the last residue. If one starts with an alkane with an odd number of carbons, the three-carbon fragment propionyl-CoA will be the last residue. Propionyl-CoA is then converted to succinyl-CoA, a four-carbon molecule that is an intermediate of the TCA cycle.

What types of alkanes do microbes most prefer? In general, midsize straight-chain aliphatics (*n*-alkanes C_{10} to C_{18} in length) are utilized more readily than *n*-alkanes with either shorter or longer chains. Long-chain *n*-alkanes are utilized more slowly because of low bioavailability resulting from extremely low water solubilities (Miller and Bartha, 1989). For example, the water solubility of decane (C_{10}) is 0.052 mg/l, and the solubility of octadecane (C_{18}) is almost 10-fold less (0.006 mg/l). Solubility continues to decrease with increasing chain length. In contrast, short-chain *n*-alkanes have higher aqueous solubility, e.g., the water solubility of butane (C_4) is 61.4 mg/l, but they are toxic to cells. Short-chain alkanes are toxic to microorganisms because their increased water solubility results in increased uptake of the alkanes, which are then dissolved in the cell membrane. The presence of these short alkanes within the cell membrane can alter the fluidity and integrity of the cell membrane.

The toxicity of short-chain *n*-alkanes can be mediated in some cases by the presence of free phase oil droplets. Protection occurs because the short-chain alkanes partition into the oil droplets. This results in reduced bioavailability because the aqueous phase concentration is decreased. Thus, *n*-alkane degradation rates will differ depending on whether the substrate is present as a pure compound or in a mixture of compounds.

Biodegradability of aliphatics is also negatively influenced by branching in the hydrocarbon chain. The degree of resistance to biodegradation depends on both the number of branches and the positions of methyl groups in the molecule. Compounds with a quaternary carbon atom (four carbon–carbon bonds) such as that shown in Fig. 16.10B are extremely stable because of steric effects, as discussed in Section 16.4.3.1.

Alkenes

Alkenes are hydrocarbons that contain one or more double bonds. The majority of alkene biodegradability studies have used 1-alkenes as model compounds (Britton, 1984). These studies have shown that alkenes and alkanes have comparable biodegradation rates. As illustrated in Fig. 16.15, the initial step in 1-alkene degradation can involve attack at the terminal (1) or a

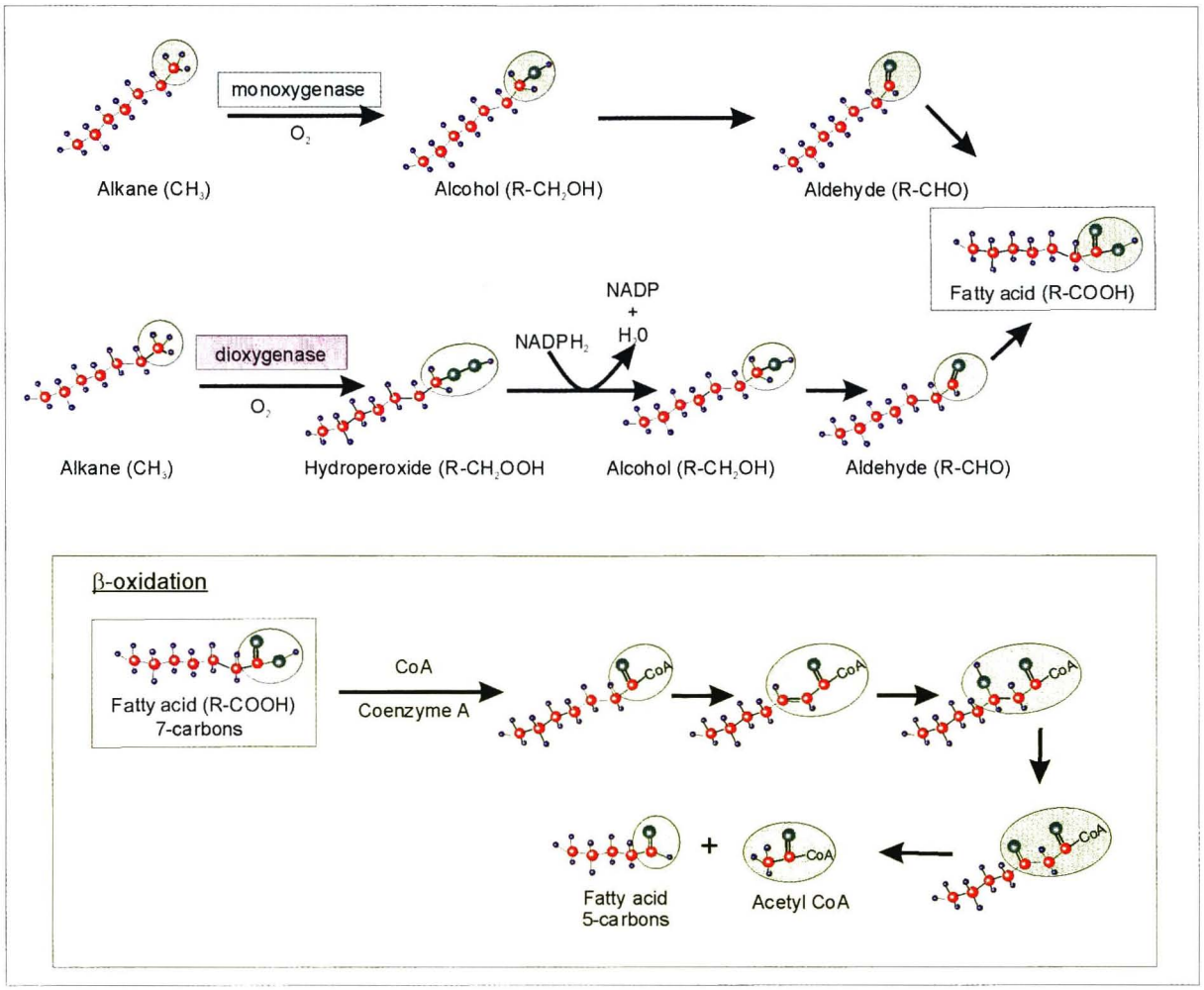


FIGURE 16.14 Biodegradation of alkanes.

subterminal (2) methyl group as described for alkanes. Alternatively, the initial step can be attack at the double bond, which can yield a primary (3) or secondary alcohol (4) or an epoxide (5). Each of these initial degradation products is further oxidized to a primary fatty acid, which is degraded by β-oxidation as shown in Fig. 16.14 for alkanes.

Halogenated Aliphatics

Chlorinated solvents such as trichloroethylene (TCE) have been extensively used as industrial solvents. As a result of improper use and disposal, these solvents are among the most frequently detected types of organic contaminants in groundwater (U.S. EPA, 1984). The need for efficient and cost-effective remediation of solvent-contaminated sites has stimulated interest in the biodegradation of these C_1 and C_2 halogenated aliphatics. Halogenated aliphatics are generally degraded more slowly than aliphatics without halogen substitution. For example, although 1-chloroalkanes ranging from C_1 to C_{12} are degraded as a sole source of carbon and energy in pure culture, they are degraded more slowly than their nonchlorinated counterparts. The presence of two or three chlorines bound to the same carbon atom inhibits aerobic degradation (Jannsen *et al.*, 1990). Further, degradation rates of 1-chloroalkanes, ranging from C_3 to C_{12}, increased with increasing alkyl chain length (Okey and Bogan, 1965). These results can be explained by the decreasing electronic effects of the chlorine atom on the enzyme–carbon reaction center as the alkane chain length increases (see Section 16.4.3.2).

Biodegradation of halogenated aliphatics occurs by three basic types of reactions. **Substitution** is a nucleophilic reaction (the reacting species brings an electron pair) in which the halogens on a mono- or dihalogenated compound are substituted by a hydroxy group (Fig. 16.16). **Oxidation** reactions are catalyzed by a

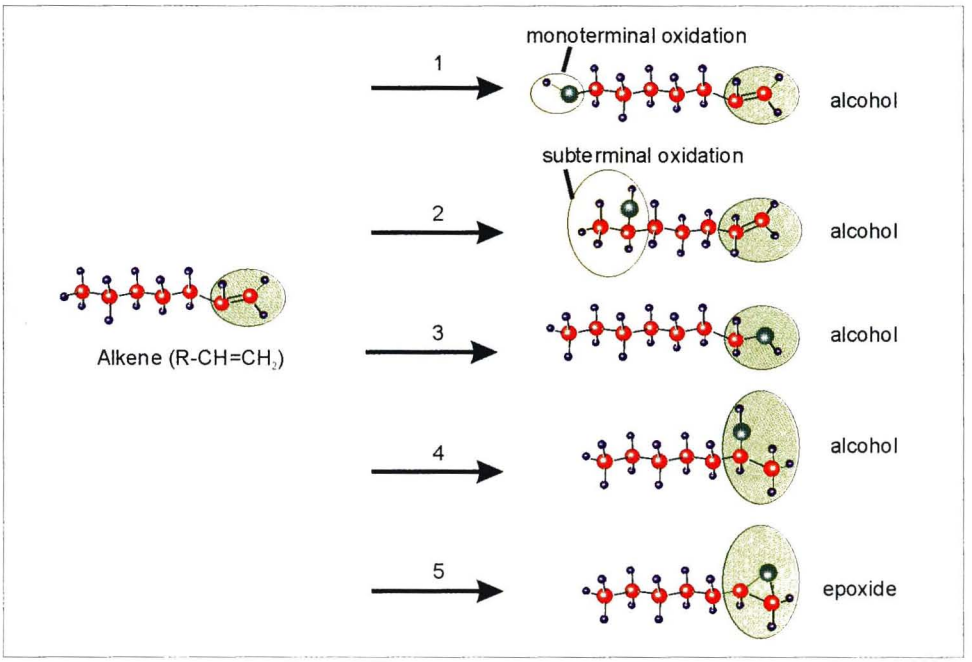


FIGURE 16.15 Biodegradation of alkenes.

select group of monooxygenase and dioxygenase enzymes that have been reported to oxidize highly chlorinated C_1 and C_2 compounds, e.g., TCE. These mono- and dioxygenase enzymes are produced by bacteria to oxidize a variety of nonchlorinated compounds including methane, ammonia, toluene, and propane. These enzymes do not have exact substrate specificity, and thus they can also participate in the cometabolic degradation of chlorinated aliphatics (Section 16.3, Fig. 16.5). Usually, a large ratio of substrate to chlorinated aliphatic is required to achieve cometabolic degradation of the chlorinated aliphatic. Reported examples of oxygenase–substrate systems that cometabolically degrade chlorinated hydrocarbons include methane monooxygenase produced by methanotrophic bacteria during growth on carbon sources such as methane or formate, toluene dioxygenase produced during growth of some bacteria on toluene, ammonia monooxygenase produced by *Nitrosomonas europaea* during growth on ammonia (Vannelli *et al.*, 1990), and propane monooxygenase produced by *Mycobacterium vaccae* JOB5 during growth on propane (Wackett *et al.*, 1989). Figure 16.17 shows an example of cometabolic oxidation of a C_1 compound, chloroform (see pathway 1), and a C_2 alkene, TCE (see pathway 2).

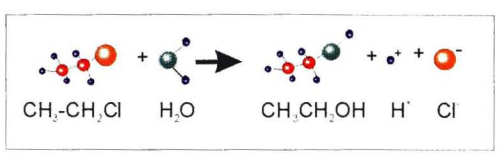


FIGURE 16.16 Dechlorination by substitution.

Reductive dehalogenation, the third type of reaction involved in biodegradation of halogenated organics, is mediated by reduced transition metal complexes. Reductive dehalogenation generally occurs in an anaerobic environment. However, the second of the reactions shown in Fig. 16.18, formation of alkenes, can occur aerobically for a limited number of chlorinated compounds that have a higher reducing potential than O_2, e.g., hexachloroethane and dibromoethane (Vogel *et al.*, 1987). In the first step of reductive dehalogenation, electrons are transferred from the reduced metal to the halogenated aliphatic, resulting in an alkyl radical and free halogen. The alkyl radical can either scavenge a hydrogen atom (1) or lose a second halogen to form an alkene (2).

Generally, aerobic conditions favor the biodegradation of compounds with fewer halogen substituents, and anaerobic conditions favor the biodegradation of compounds with a high number of halogen substituents. However, complete biodegradation of highly halogenated aliphatics under anaerobic conditions often does not take place. Therefore, some researchers have proposed the use of a sequential anaerobic and aerobic treatment (Fathepure and Vogel, 1991). Initial incubation under anaerobic conditions would be used to decrease the halogen content, and subsequent addition of oxygen would create aerobic conditions to allow complete degradation to proceed aerobically.

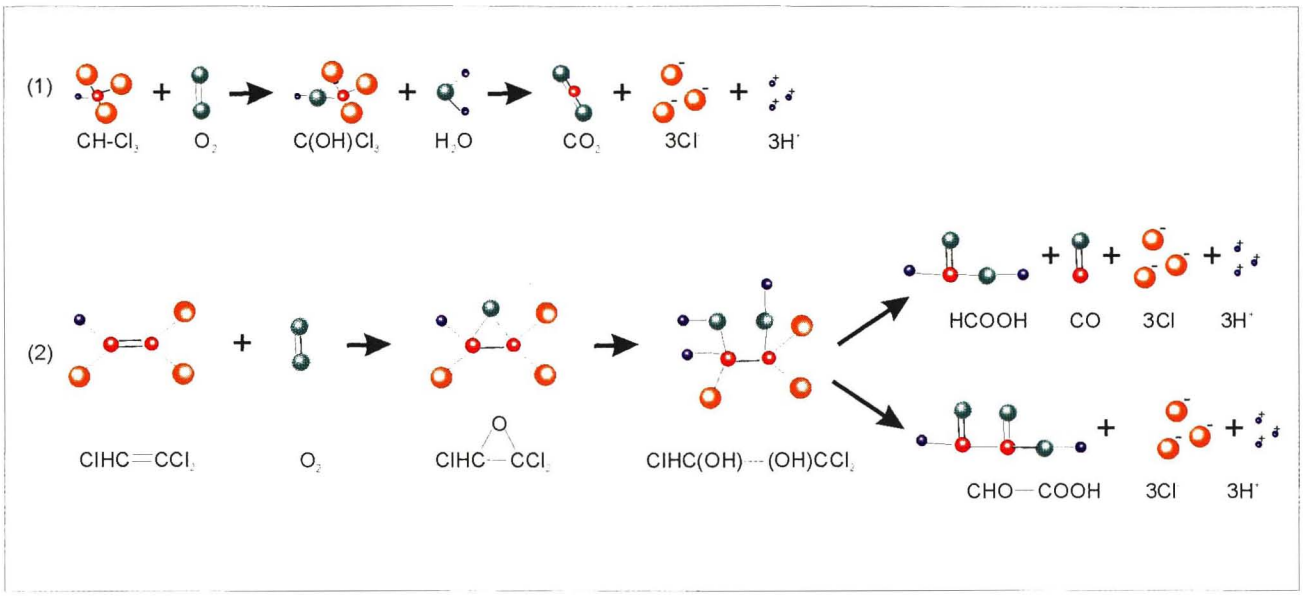


FIGURE 16.17 Dehalogenation by oxidation.

One important consideration in the bioremediation of sites containing chlorinated aliphatics is the potential toxicity of such compounds. For example, the effect of solvent concentration on inhibition of a propylene-grown *Xanthobacter* strain capable of oxidizing several chlorinated alkenes, including TCE, was determined (Ensign *et al.*, 1992). It was found that alkene monooxygenase activity was up to 90% inhibited by chlorinated alkene concentrations between 25 and 330 μM, depending on the compound. Similarly, it was found that toluene used to support the aerobic metabolism of TCE in soil could be inbitory (Mu and Scow, 1994). Specifically, increasing the TCE concentration from 1 to 20 μg/ml decreased the numbers of toluene and TCE degraders in the soil and decreased the rate of TCE degradation. Also of concern is that TCE transformations can result in oxidized derivatives more toxic than TCE itself. This toxicity has been attributed to a nonspecific binding of TCE transformation products to cellular proteins. For example, TCE transformation by methane monooxygenase results in a transformation product that can bind to a protein subunit of the monooxygenase enzyme, resulting in inhibition of enzyme activity (Oldenhuis *et al.*, 1991). Recovery of oxidizing activity is possible by replacement of the enzyme following *de novo* protein synthesis. Similar behavior was found for ammonia monoxy-genase. In this experiment, a wide variety of

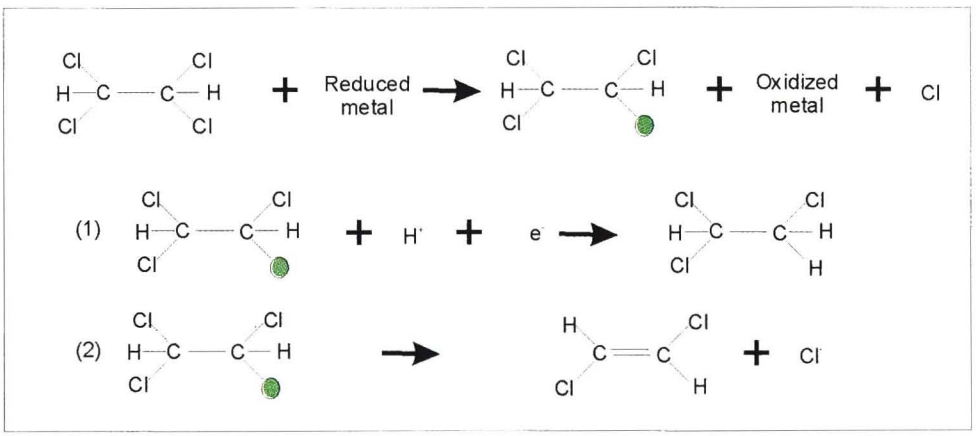


FIGURE 16.18 Reductive dehalogenation of tetrachloroethane to trichloroethane (1) or dichloroethylene (2).

chlorinated aliphatic compounds were tested and it was found that ammonia monooxygenase activity was strongly inhibited after the oxidation of some chlorinated ethylenes and compounds containing a dichlorinated carbon (Rasche *et al.*, 1991). These examples all demonstrate that harnessing cometabolic activity for unusual substrates is a complex process. Not only does one have to understand the cometabolic reaction, one must also consider the potential toxicity of the cometabolic substrate and its metabolites to degrading microbes.

16.6.2.2 Alicyclics

Alicyclic hydrocarbons (Fig. 16.13) are major components of crude oil, 20 to 70% by volume. They are commonly found elsewhere in nature as components of plant oils and paraffins, microbial lipids, and pesticides (Trudgill, 1984). The various components can be simple, such as cyclopentane and cyclohexane, or complex, such as trimethylcyclopentane and various cycloparaffins. The use of alicyclic compounds in the chemical industry, and the release of alicyclics to the environment through industrial processes, other than oil processing and utilization, is more limited than for aliphatics and aromatics. Consequently, the issue of health risks associated with human exposure to alicyclics has not reached the same level of importance as for the other classes of compounds, especially the aromatics. As a result, far less research has focused on the study of alicyclic biodegradation.

It is known that there is no correlation between the ability to utilize *n*-alkanes and the ability to oxidize cycloalkanes fully. Further, it is difficult to isolate pure cultures that degrade alicyclic hydrocarbons using enrichment techniques. Although microorganisms with complete degradation pathways have been isolated (Trower *et al.*, 1985), alicyclic hydrocarbon degradation is thought to occur primarily by commensalistic and cometabolic reactions as shown for cyclohexane in Fig. 16.19. In this series of reactions, one organism converts cyclohexane to cyclohexanone via a cyclohexanol (step 1 and step 2), but is unable to lactonize and open the ring. A second organism that is unable to oxidize cyclohexane to cyclohexanone can perform the lactonization, ring opening, and mineralization of the remaining aliphatic compound (Perry, 1984).

Cyclopentane and cyclohexane derivatives that contain one or two OH, C=O, or COOH groups are readily metabolized, and such degraders are easily isolated from environmental samples. In contrast, degradation of alicyclic derivatives containing one or more CH_3 groups is inhibited. This is reflected in the decreasing rate of biodegradation for the following series of alkyl derivatives of cyclohexanol: cyclohexanol > methylcyclohexanol > dimethylcyclohexanol (Pitter and Chudoba, 1990).

16.6.2.3 Aromatics

Unsubstituted Aromatics

Aromatic compounds contain at least one unsaturated ring system with the general structure C_6R_6, where R is any functional group (Fig. 16.13). Benzene (C_6H_6) is the parent hydrocarbon of this family of unsaturated cyclic compounds. Compounds containing two or more fused benzene rings are called polyaromatic hydrocarbons (PAH). Aromatic hydrocarbons are natural products; they are part of lignin and are formed as organic materials are burned, for example, in forest fires. However, the addition of aromatic compounds to the environment has increased dramatically through activities such as fossil fuel processing and

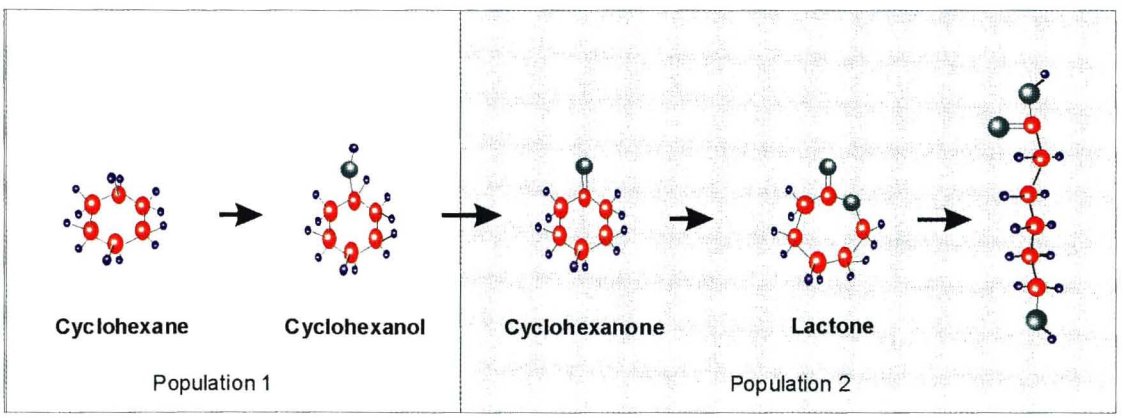


FIGURE 16.19 Degradation of cyclohexane.

utilization and burning of wood and coal (Cerniglia and Heitkamp, 1989). The quantity and composition of the aromatic hydrocarbon component in petroleum products are of major concern when evaluating a contaminated site because several components of the aromatic fraction have been shown to be carcinogenic to humans. Aromatic compounds also have demonstrated toxic effects toward microorganisms. For example, the toxicity of the water-soluble fraction of refined oil is more toxic to the growth of heterotrophic microorganisms than the water soluble fraction of crude oil. This is attributed to the greater proportion of aromatic compounds, particularly naphthalene and alkylnaphthalenes, present in refined oils compared with crude oils. For example, the total naphthalene concentration of the refined oil was 1800 μg/l, compared with only 45 μg/l in the crude oil tested (Hodson *et al.*, 1977). Autotrophs are also affected adversely. When the impact of the aliphatic, aromatic, and asphaltic fractions from five petroleum oils on photosynthesis and respiration of a representative cyanobacterium was determined, it was found that growth inhibition was most strongly associated with the aromatic fraction (Singh and Guar, 1990).

Because of the potential human health impacts of aromatic compounds, their biodegradation has been extensively studied (Gibson and Subramanian, 1984). The results of this work showed that a wide variety of bacteria and fungi can carry out aromatic transformations, both partial and complete, under a variety of environmental conditions. Under aerobic conditions, the most common initial transformation is a hydroxylation that involves the incorporation of molecular oxygen. The enzymes involved in these initial transformations are either monooxygenases or dioxygenases. In general, prokaryotic microorganisms transform aromatics by an initial dioxygenase attack to *cis*- dihydrodiols. The *cis*-dihydrodiol is rearomatized to form a dihydroxylated intermediate, catechol. The catechol ring is cleaved by a second dioxygenase either between the two hydroxyl groups **(ortho pathway)** or next to one of the hydroxyl groups **(meta pathway)** and further degraded to completion (Fig. 16.20).

Eukaryotic microorganisms initially attack aromatics with a cytochrome P-450 monooxygenase, incorporating one atom of molecular oxygen into the aromatic and reducing the second to water, resulting in the formation of an arene oxide. This is followed by the enzymatic addition of water to yield a *trans*-dihydrodiol (Fig. 16.21). Alternatively, the arene oxide can be isomerized to form phenols, which can be conjugated with sulfate, glucuronic acid, and glutathione. These conjugates are similar to those formed in higher organisms and seem to aid in detoxification and elimination of aromatic compounds. The exception to this is the white-rot fungi which under certain conditions are able to completely mineralize aromatic compounds (see Case Study 1).

Thus far, six families of genes responsible for PAH degradation have been identified. Often the capacity for aromatic degradation, especially chlorinated aromatics, is plasmid mediated (Ghosal *et al.*, 1985). Plasmids can carry both individual genes and operons encoding partial or complete biodegradation of an aromatic compound. An example of a plasmid that carries a family of genes involved in the degradation of aromatic compounds is the NAH7 plasmid, which codes for the degradation of naphthalene. The NAH7 plasmid was obtained from *Pseudomonas putida* and contains genes that encode the enzymes for the first 11 steps of naphthalene oxidation. This plasmid or closely related plasmids are frequently found in sites that are contaminated with PAHs (Ahn *et al.*, 1999). As discussed in Chapter 13, this plasmid has also been used to construct a luminescent bioreporter gene system (Fig. 13.20). Here the lux genes that cause luminescence have been inserted into the *nah* operon in the NAH plasmid. When the *nah* operon is induced by the presence of naphthalene, both naphthlene-degrading genes and the lux gene are expressed. As a result, naphthalene is degraded and the reporter organism luminesces. Such reporter organisms are currently being used to study the effect of oxygen and substrate level on degradation of naphthalene in soil systems.

There is also interest in construction of bacterial strains with a broad aromatic biodegradation potential. Although it is possible to manipulate the chromosome, plasmids offer an easy mechanism by which new genes can be introduced into a bacterial cell.

In general, aromatics composed of one, two, or three condensed rings are transformed rapidly and often completely mineralized, whereas aromatics containing four or more condensed rings are transformed much more slowly, often as a result of cometabolic attack. This is due to the limited bioavailability of these high-molecular-weight aromatics. Such PAHs have very limited aqueous solubility and sorb strongly to particle surfaces in soil and sediments. However, it has been demonstrated that chronic exposure to aromatic compounds will result in increased transformation rates because of adaptation of an indigenous population to growth on aromatic compounds.

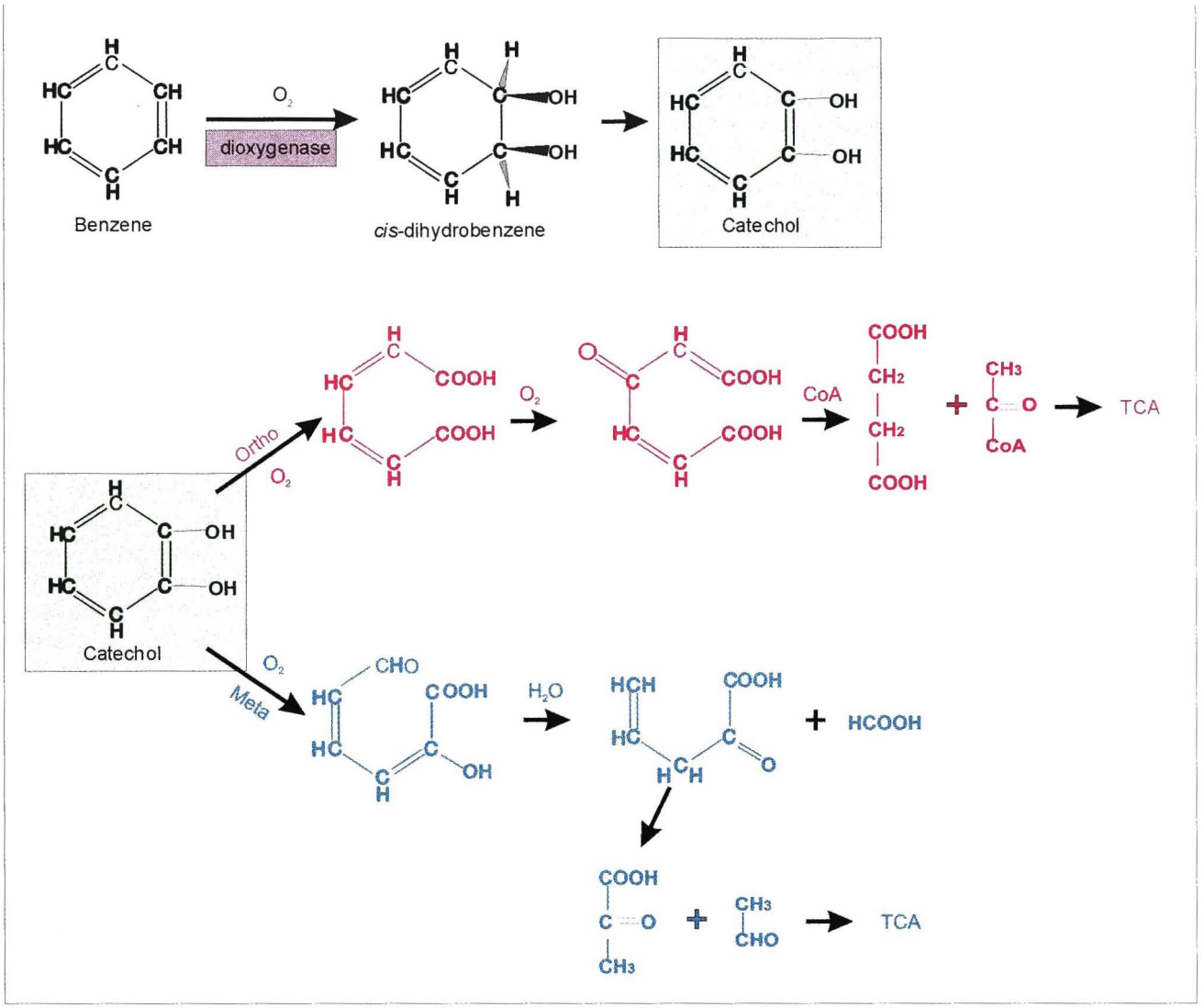


FIGURE 16.20 Incorporation of oxygen into the aromatic ring by the dioxygenase enzyme, followed by meta or ortho ring cleavage.

Substituted Aromatics

One group of aromatics of special interest is the **chlorinated aromatics.** These compounds have been used extensively as solvents and fumigants (e.g., dichlorobenzene), and wood preservatives (e.g., pentachlorophenol (PCP)) and are parent compounds for pesticides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and DDT. The difficulty for microbes in the degradation of chlorinated organics is that the carbon–chlorine bond is very strong and requires a large input of energy to break. Second, the common intermediate in aromatic degradation is catechol or dihydroxybenzene (see Fig. 16.20). This molecule requires two adjacent unsubstituted carbons so that hydroxyl groups can be added. Chlorine substituents can block these sites. Some strategies for degradation of chlorinated aromatics are shown in Fig. 16.22 for pentachlorophenol and dichlorobenzene.

Chlorinated phenols are particularly toxic to microorganisms. In fact, phenol itself is very toxic and is used as a disinfectant. Chlorination adds to toxicity, which increases with the degree of chlorination (Chaudhry and Chapalamadugu, 1991). For example, Van Beelen and Fleuren-Kemilä (1993) quantified the effect of PCP and several other pollutants on the ability of soil microorganisms to mineralize [^{14}C]acetate in soil. The amount of PCP required to reduce the initial rate of acetate mineralization by 10% ranged between

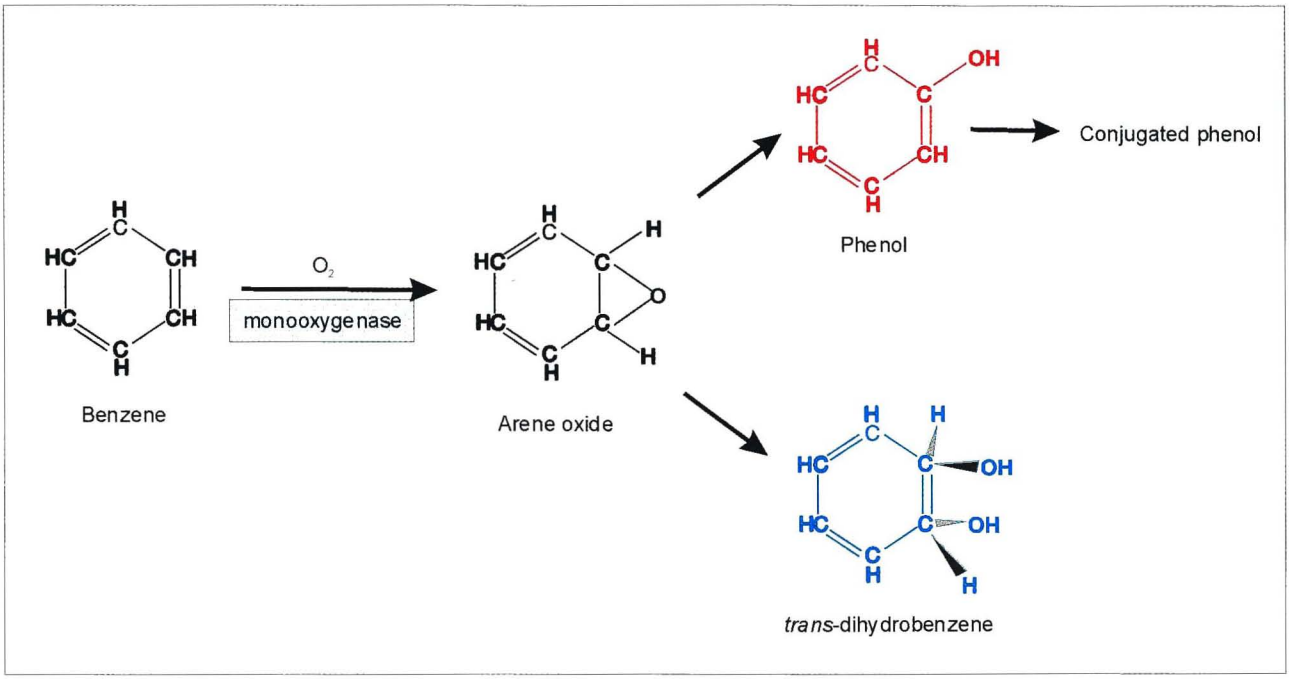


FIGURE 16.21 Fungal monooxygenase incorporation of oxygen into the aromatic ring.

0.3 and 50 mg/kg dry soil, depending on the soil type. High concentrations of PCP also have inhibitory effects on PCP-degrading microorganisms. For example, Alleman *et al.* (1992) investigated the effect of PCP on six species of PCP-degrading fungi. They showed that increasing the PCP concentration from 5 to 40 mg/l decreased fungal growth and decreased the ability of the fungi to degrade PCP.

Methylated aromatic derivatives, such as toluene, constitute another common group of substituted aromatics. These are major components of gasoline and are commonly used as solvents. These compounds can initially be attacked either on the methyl group or directly on the ring as shown in Fig. 16.23. **Alkyl** derivatives are attacked first at the alkyl chain, which is shortened by β-oxidation to the corresponding benzoic acid or phenylacetic acid, depending on the number of carbon atoms. This is followed by ring hydroxylation and cleavage (Fig. 16.23).

Dioxins

Dioxins and dibenzofurans are created during waste incineration and are part of the released smoke stack effluent. Once thought to be one of the most potent carcinogens known, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is associated with the manufacture of 2,4-D and 2,4,5-trichlorophenoxy acetic acid (2,4,5-T), hexacholorophene, and other pesticides that have 2,4,5-T as a precursor. Current thinking is that TCDD is less dangerous in terms of carcinogenicity and teratogenicity than once thought, but that noncancer risks including diabetes, reduced IQ, and behavioral impacts may be more important. The structure of TCDD (Fig. 16.13) and its low water solubility, 0.002 mg/l, result in great stability of this molecule in the environment. Although bacterial and fungal biodegradation of TCDD has been demonstrated, the extent of biodegradation is very minimal. For example, a mixture of six bacterial strains isolated from TCDD-contaminated soil obtained from Seveso, Italy was able to produce a metabolite presumed to be 1-hydroxy-TCDD (Phillippi *et al.*, 1982). However, less than 1% of the original TCDD was degraded in 12 weeks. In addition, the fungus *Phanerochaete chrysosporium* (see Case Study 1) was only able to mineralize 2% of the parent compound to CO_2 (Bumpus *et al.*, 1985). More recent work has focused on the reductive dechlorination of TCDD and more highly chlorinated isomers.

Heterocyclic Compounds

Heterocyclics are cyclic compounds containing one or more heteroatoms (nitrogen, sulfur, or oxygen) in addition to carbon atoms. The dioxins already discussed as well as other compounds shown in Fig. 16.13 fall into this category. In general, heterocyclic compounds are more difficult to degrade than analogous

CASE STUDY 1

This chapter is focused primarily on the bacterial transformations of organic contaminants. This case study is included to emphasize that other microbes can also participate in the biodegradation process. One class of microbes of particular interest with respect to degradation of aromatic contaminants is the white rot fungi. These fungi are important wood degraders and utilize both the lignin and cellulose components of wood. A by-product of the degradation process is a white fibrous residue, hence the name "white rot". Recall that the lignin structure is based on two aromatic amino acids, tyrosine and phenylalanine (Figure 14.5F). In order to degrade an amorphous aromatic-based structure such as lignin, the white rot fungi release a nonspecific extracellular enzyme, H_2O_2-dependent lignin peroxidase, that is used in conjunction with an extracellular oxidase enzyme that generates H_2O_2. This enzyme-H_2O_2 system generates oxygen-based free radicals that react with the lignin polymer to release residues that are taken up by the cell and degraded. Since the lignin structure is based on an aromatic structure and the initial enzymes used to degrade lignin are nonspecific, the white rot fungi are able to degrade a variety of aromatic contaminants. The most famous of the white rot fungi is *Phanerochaete chrysosporium*, which has been demonstrated to degrade a variety of aromatic compounds including the pesticide DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane), the herbicides 2,4-D and 2,4,5-T (chlorophenoxyacetates), the wood preservative PCP (pentachlorophenol), PCBs (polychlorinated biphenyls), PAHs (polyaromatic hydrocarbons), and chlorinated dioxins (Glaser and Lamar, 1995; Hammel, 1995).

It should be noted that *P. chrysosporium* grown under nitrogen-rich conditions behaves like other eukaryotic microbes, it produces trans-dihydrodiols (see Figure 16.21) and various conjugates that are excreted. Under these conditions aromatic contaminant molecules are not degraded. However, under nitrogen-limiting conditions, which induce production of lignin-degrading enzymes, aromatic contaminants are mineralized. The application of white-rot fungi and in particular *P. chrysosporium* to problems in Environmental Microbiology is being investigated from several aspects. These include the use of the fungus to delignify lignocellulosic materials that would normally be considered as waste, e.g. straw, to produce cattle feed (Carlile and Watkinson, 1994). These fungi can also be used to delignify wood pulp that is used in paper manufacture. In this case a biological process replaces one that is normally done mechanically. A further application of these fungi is the degradation of aromatic components found in wastestreams associated with the paper industry (a variety of unchlorinated and chlorinated aromatic components), wood treatment industry (PCP and various creosote components), and the munitions industry (TNT (2,4,6-trinitrotoluene) and DNT (2,4-dinitrotoluene)).

aromatics that contain only carbon. This is probably due to the higher electronegativity of the nitrogen and oxygen atoms compared with the carbon atom, leading to deactivation of the molecule toward electrophilic substitution. Heterocyclic compounds with five-membered rings and one heteroatom are readily biodegradable, probably because five-membered ring compounds exhibit higher reactivity toward electrophilic agents and are hence more readily biologically hydroxylated. The susceptiblity of heterocyclic compounds to biodegradation decreases with increasing number of heteroatoms in the molecules.

16.6.2.4 Pesticides

Pesticides are the biggest nonpoint source of chemicals added to the environment. The majority of the currently used organic pesticides are subject to extensive mineralization within the time of one growing season or less. Synthetic pesticides show a bewildering variety of chemical structures, but most can be traced to relatively simple aliphatic, alicyclic, and aromatic base structures already discussed. These base structures bear a variety of halogen, amino, nitro, hydroxyl, carboxyl, and phosphorus substituents. For example, the chlorophenoxyacetates, such as 2,4-D and 2,4,5-T, have been released into the environment as herbicides over the past 40 years. Both of these structures are biodegradable and pathways are presented in Fig. 16.24.

As an exercise, examine the pesticide structures presented in Fig. 16.25. For each set of pesticides, predict which is more easily degraded. You are correct if you predicted 2,4-D for the first set. It is rapidly degraded by soil microorganisms. Although 2,4,5-T is also degraded, the degradation is much slower. For the second set of pesticides, propham is more degradable. In

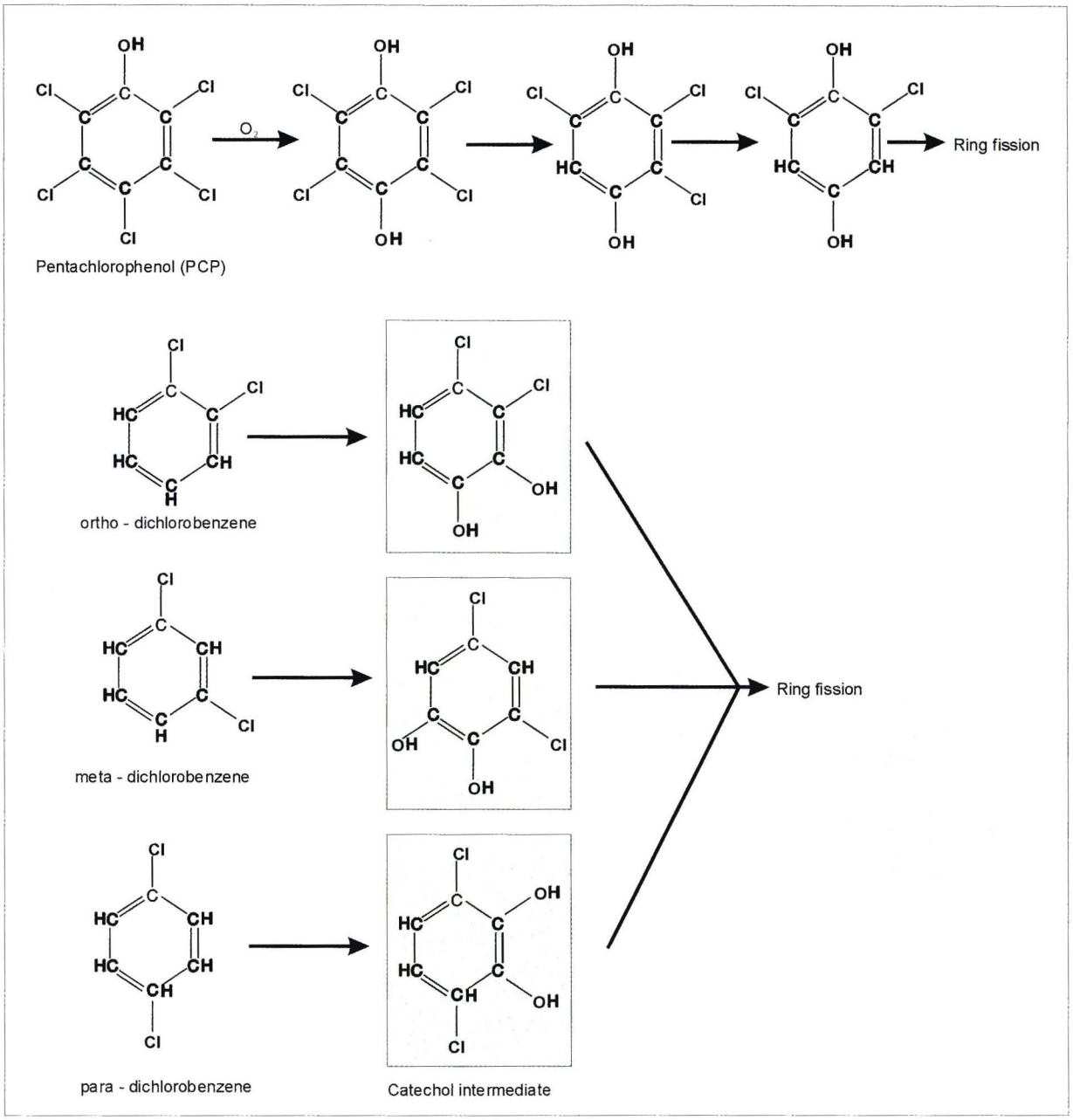


FIGURE 16.22 Initial steps in the aerobic degradation of pentachlorophenol and three dichlorobenzenes.

propachlor, the extensive branching so close to the ring structure blocks biodegradation. In the third set, carbaryl is more degradable because of the extensive chlorination and complex ring structures of aldrin. In fact, the estimated **half-life** of carbaryl in soil is 30 days, compared with 1.6 years for aldrin. Half-life is a term used to express the time it takes for 50% of the compound to disappear. Generally, 5½ half-lives are believed sufficient for the compound to be completely degraded. Finally, in the fourth set, methoxychlor is more degradable than DDT. In this case, the half-lives are even longer, 1 year for methoxychlor and 15.6 years for DDT.

16.6.3 Anaerobic Conditions

Anaerobic conditions are not uncommon in the environment. Most often, such conditions develop in water or saturated sediment environments. But, even in well-aerated soils there are microenvironments with little or no oxygen. In all of these environments, **anaerobiosis** occurs. Anaerobiosis occurs when the rate of

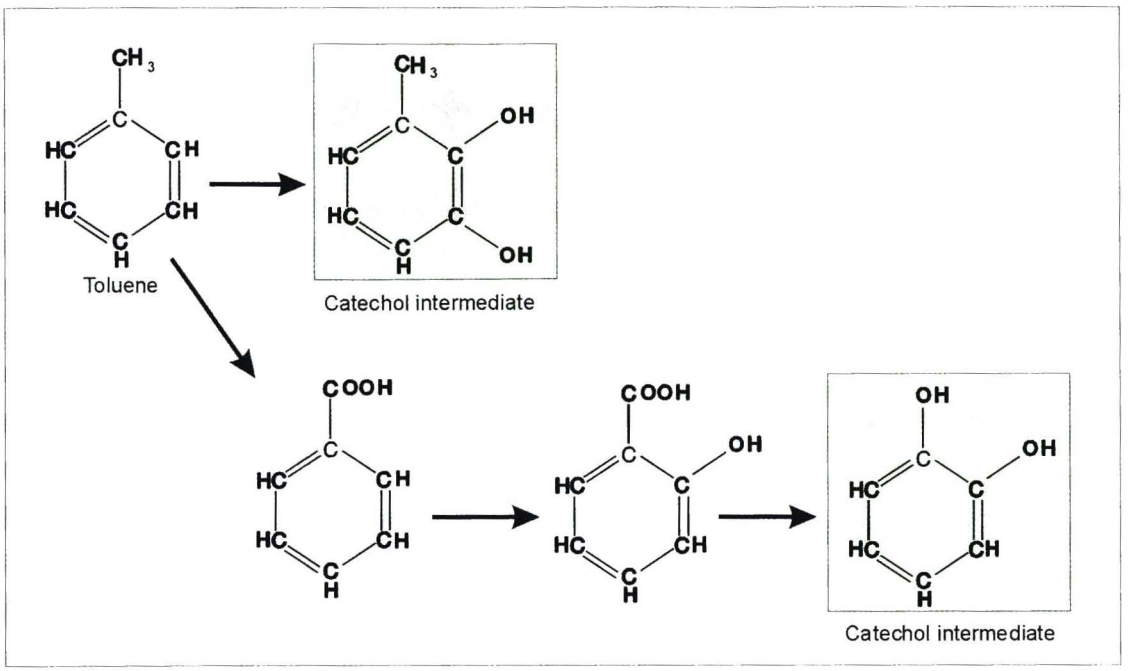


FIGURE 16.23 Biodegradation of toluene.

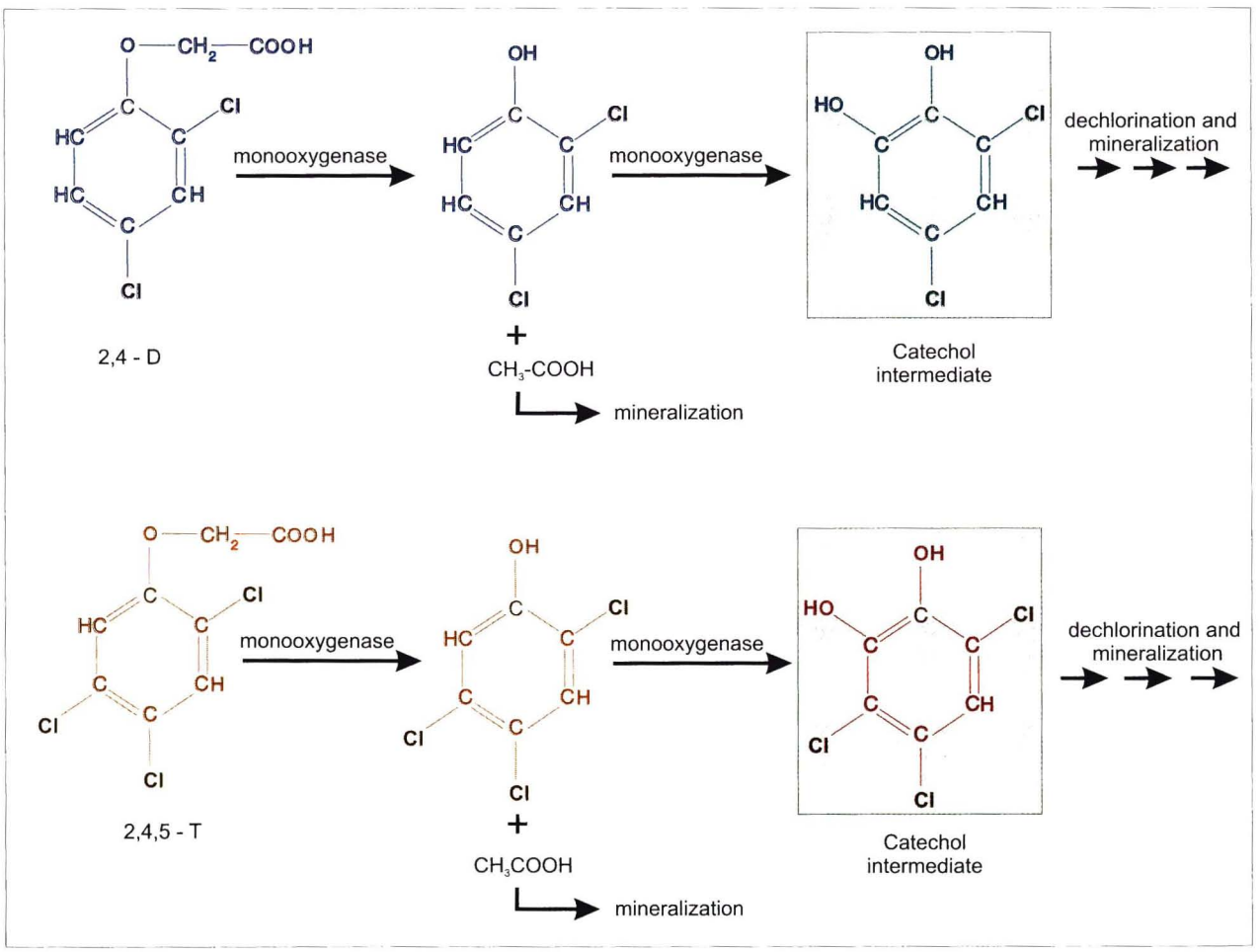


FIGURE 16.24 Biodegradation of 2,4-D and 2,4,5-T.

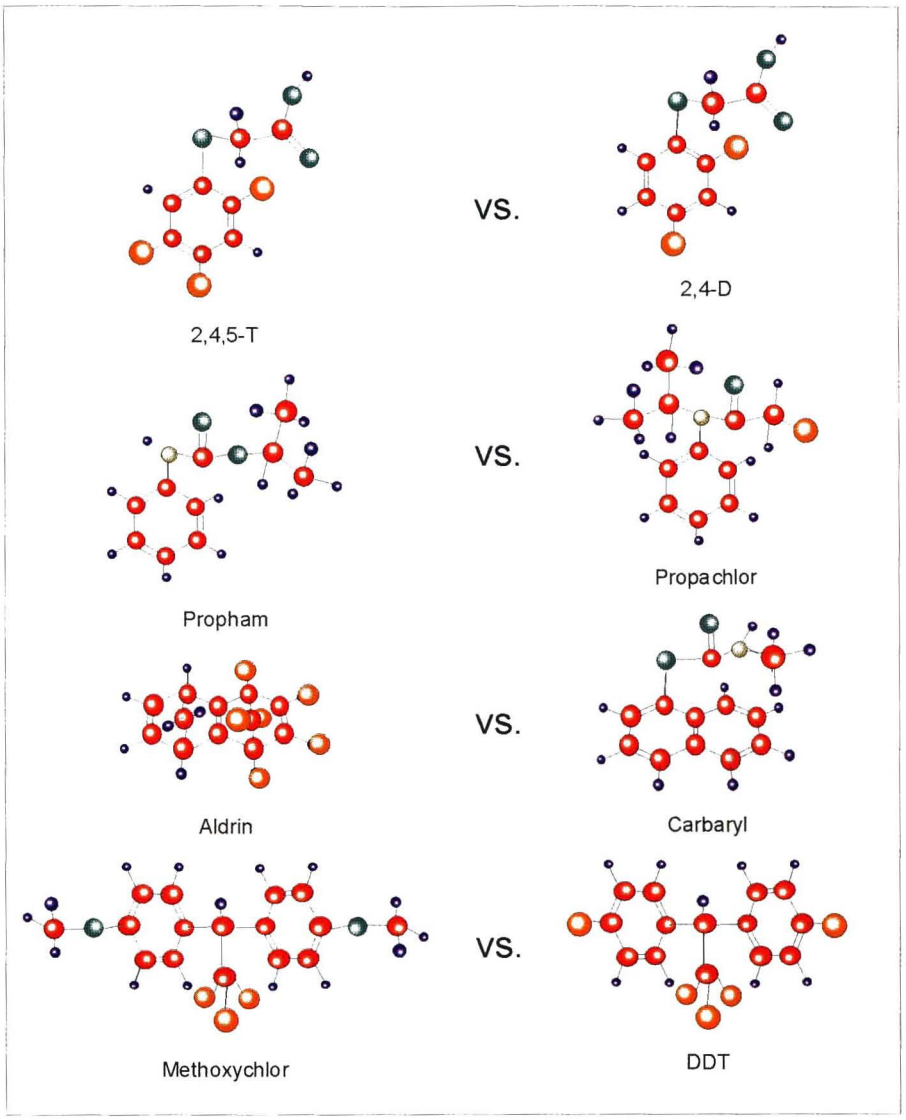


FIGURE 16.25 Comparison of four sets of pesticides. Can you predict which of each set is more easily biodegraded?

oxygen consumption by microorganisms is greater than the rate of oxygen diffusion through either air or water. In the absence of oxygen, organic compounds can be mineralized through anaerobic respiration using a terminal electron acceptor other than oxygen (see Chapter 3.4). There is a series of alternative terminal electron acceptors in the environment including iron, nitrate, manganese, sulfate, and carbonate. These alternative electron acceptors have been listed in the order of most oxidizing to most reducing, and are usually utilized in this order, because the amount of energy generated for growth depends on the oxidation potential of the electron acceptor. Because none of these electron acceptors are as oxidizing as oxygen, growth under anaerobic conditions is never as efficient as growth under aerobic conditions.

16.6.3.1 Aliphatics

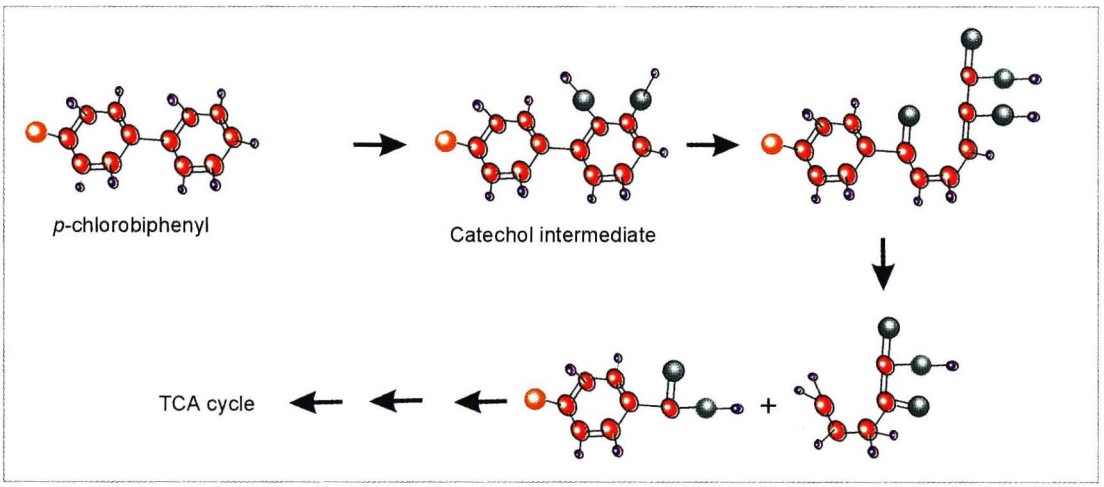
Saturated aliphatic hydrocarbons are degraded slowly if at all under anaerobic conditions. This is supported by the fact that hydrocarbons in natural underground reservoirs of oil (which are under anaerobic conditions) are not degraded despite the presence of microorganisms. However, both unsaturated aliphatics and aliphatics containing oxygen (aliphatic alcohols and ketones) are readily biodegraded anaerobically. The suggested pathway of biodegradation for unsaturated hydrocarbons is hydration of the double bond to an alcohol, with further oxidation to a ketone or aldehyde and, finally, formation of a fatty acid (Fig. 16.26).

As previously discussed, halogenated aliphatics can be partially or completely degraded under anaerobic

CASE STUDY 2

Polychlorinated Biphenyls

Biphenyl is the unchlorinated analogue or parent compound of the polychlorinated biphenyls (PCBs), which were first described in 1881 (Waid 1986a; 1986b). The PCBs consist of variously chlorine-substituted biphenyls of which, in theory, there are 209 possible isomers. Only approximately 100 actually exist in commercial formulations. The aqueous solubility of biphenyl is 7.5 mg/l, and any chlorine substituent decreases the water solubility. In general, the water solubility of monochlorobiphenyls ranges from 1 to 6 mg/l, for dichlorobiphenyls the range is 0.08–5 mg/l, and for hexachlorobiphenyl the aqueous solubility is just 0.00095 mg/l. By 1930, because of their unusual stability, PCBs were widely used as nonflammable heat-resistant oils in heat transfer systems, as hydraulic fluids and lubricants, as transformer fluids, in capacitors, as plasticizers in food packaging materials, and as petroleum additives. PCBs were used as mixtures of variously chlorinated isomers and marketed under various trade names, e.g., Aroclor (U.S.), Clophen (West Germany), Phenoclor (Italy), Kanechlor (Japan), Pyralene (France), and Soval (U.S.S.R.). The U.S. domestic sales of Aroclors 1221–1268 (where the last two numbers indicated the % chlorination) went from 32 million pounds in 1957 to 80 million pounds in 1970, with the most popular blend being 1242. PCB use in transformers and capacitors accounted for about 50% of all Aroclors used.



A rice oil factory accident in Japan in 1968 brought PCBs international attention. In the factory, the heat exchanger pipes used to process rice oil contained PCBs as the heat exchange fluid. Unnoticed, a heat exchange pipe broke and leaked PCBs into a batch of rice oil, which was then packaged and consumed by the local population. The contaminated rice oil poisoned over 1000 people, producing a spectrum of symptoms including chloracne, gum and nail bed discoloration, joint swelling, emission of waxy secretions from eyelid glands, and lethargy. As a result, the U.S. Food and Drug Administration (FDA) issued tolerance levels for PCBs in food and packaging products, and the Environmental Protection Agency (EPA) under TOSCA, issued rules governing the use of PCBs. This has drastically reduced domestic production and use of PCBs.

Despite decreased use, PCBs still pose an environmental problem because they are not only chemically stable, they also resist biodegradation. Because past use of PCBs has been high, PCBs have accumulated in the environment. There are several ways in which PCBs make their way into the environment. One of these is in runoff from industrial waste dumps and spills. Other sources include points of PCB manufacture and processing into other products. Because PCB input into fresh water has been high in the past, PCBs have accumulated in sediments. Even if PCB input were stopped completely, previously contaminated sediments could continue to release PCBs into freshwater systems for years to come.

Although some PCB degradation occurs, it is limited by low bioavailability. The case study figure shows a typical degradation pathway. Note that the pathway includes the familiar catechol intermediate. In this example only one of the biaryl rings is chlorinated, so degradation begins with the unchlorinated ring. Not surprisingly, the number and location of chlorines help determine the rate and extent of biodegradation. This is illustrated in Table 16.2, which shows that chlorination of one of the biaryl rings does not generally inhibit degradation with the exception of the 2,6 isomer. However, chlorines on both rings inhibit degradation to some extent in most cases. This table is from an early study performed to evaluate PCB degradation. As illustrated in Table 16.2, the two degraders studied vary somewhat in their preference for certain PCB isomers. Subsequent work confirmed this finding and further found that there are bacterial isolates that actually prefer more highly chlorinated isomers to the less chlorinated ones.

The extensive research that has been performed to understand PCB degradation has suggested several strategies for promoting biodegradation. These include the use of a sequential anaerobic-aerobic process to remove chlorines and then allow mineralization of the less chlorinated isomers. A second approach is the addition of a co-substrate, such as the parent PCB compound biphenyl, to stimulate PCB-degrading populations. The rationale for this strategy is that one problem with PCB degradation is that their low bioavailability simply does not allow induction of biodegradation pathways. The addition of the biphenyl, which has a higher aqueous solubility (7.5 mg/l), can induce PCB biodegradation. Unfortunately, the addition of biphenyl to the environment is problematic because of cost and toxicity. However, it has been demonstrated that some plant compounds can also induce PCB biodegradation. One such compound is l-carvone from spearmint (Gilbert and Crowley, 1997). A third approach is **bioaugmention** or addition of specific PCB-degrading microorganisms to contaminated areas that may not contain indigenous degraders.

TABLE 16.2 Effect of Chlorine Substitution on Biodegradability of Various Polychlorinated Biphenyl Isomers

	Degradation rate (nmol/ml/hr)			Degradation rate (nmol/ml/hr)	
PCB chlorine position	*Alcaligenes sp.*	*Acinetobacter sp.*	PCB chlorine position	*Alcaligenes sp.*	*Acinetobacter sp.*
2	>50	>50	2,4,6	3.1	46.0
3	>50	>50	2,5,2′	1.6	5.1
4	>50	>50	2,5,3′	42.1	41.3
2,3	>50	46.4	2,5,4′	21.8	30.4
2,4	>50	>50	2,4,4′	41.3	40.2
2,5	>50	>50	3,4,2′	15.6	38.6
2,6	0	4.1	2,3,4,5	25.8	19.1
3,4	>50	>50	2,3,5,6	0	0
3,5	>50	>50	2,3,2′,3′	8.7	7.3
2,2′	6.3	14.0	2,4,2′,4′	0	0
2,4′	48.2	49.1	2,4,3′,4′	0	0
3,3′	—	18.5	2,5,2′,5′	0	3.5
4,4′	16.2	25.2	2,6,2′,6′	0	0
2,3,4	35.1	32.0	3,4,3′,4′	0	0
2,3,6	0	0	2,4,5,2′,5′	0.6	0
2,4,5	46.0	32.4			

From Furukawa, *et al.* (1978).

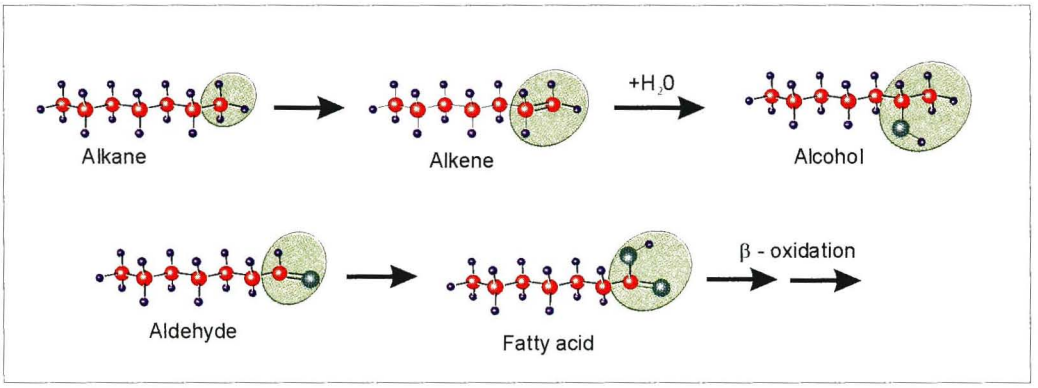


FIGURE 16.26 Anaerobic biodegradation of aliphatic compounds.

conditions by reductive dehalogenation, a cometabolic degradation step that is mediated by reduced transition metal complexes. As shown in Fig. 16.18, in the first step, electrons are transferred from the reduced metal to the halogenated aliphatic, resulting in an alkyl radical and free halogen. Then the alkyl radical can either scavenge a hydrogen atom (1) or lose a second halogen to form an alkene (2). In general, anaerobic conditions favor the degradation of highly halogenated compounds, whereas aerobic conditions favor the degradation of mono- and di-substituted halogenated compounds.

16.6.3.2 Aromatics

Like aliphatic hydrocarbons, aromatic compounds can be completely degraded under anaerobic conditions if the aromatic is oxygenated. There is also evidence that even nonsubstituted aromatics are degraded slowly under anaerobic conditions. Anaerobic mineralization of aromatics often requires a mixed microbial community that works together even though each of the microbial components requires a different redox potential. For example, mineralization of benzoate can be achieved by growing an anaerobic benzoate degrader in co-culture with a methanogen and sulfate reducer. The initial transformations in such a system are often carried out fermentatively, and this results in the formation of aromatic acids, which in turn are transformed to methanogenic precursors such as acetate, carbon dioxide, and formate. These small molecules can then be utilized by methanogens (Fig. 16.27). Such a mixed community is called a consortium. It is not known how this consortium solves the problem of requiring different redox potentials in the same vicinity in a soil system. Clearly, higher redox potentials are required for degradation of the more complex substrates such as benzoate, leaving smaller organic acid or alcohol molecules that are degraded at lower redox potentials. To ultimately achieve degradation may require that the organic acids and alcohols formed at higher redox potential be transported by diffusion or by movement with water (advection) to a region of lower redox potential. On the other hand, it may be that biofilms form on the soil surface and that redox gradients are formed within the biofilm allowing complete degradation to take place. Environmental microbiologists are actively exploring how anaerobes operate in the soil and vadose environments. Such an understanding is expected to aid in developing technologies to enhance degradation processes in anaerobic contaminated environments.

16.7 BIOREMEDIATION

The objective of bioremediation is to exploit naturally occurring biodegradative processes to clean up contaminated sites (National Research Council, 1993). There are several types of bioremediation. ***In situ* bioremediation** is the in-place treatment of a contaminated site. ***Ex situ* bioremediation** may be implemented to treat contaminated soil or water that

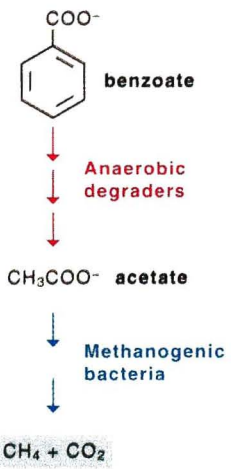


FIGURE 16.27 Anaerobic biodegradation of aromatic compounds by a consortium of anaerobic bacteria. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)

is removed from a contaminated site. **Intrinsic bioremediation** or **natural attenuation** is the indigenous level of contaminant biodegradation that occurs without any stimulation or treatment. All of these types of bioremediation continue to receive increasing attention as viable remediation alternatives for several reasons. These include generally good public acceptance and support, good success rates for some applications, and a comparatively low cost of bioremediation when it is successful. As with any technology, there are also drawbacks. Success can be unpredictable because a biological system is being used. A second consideration is that bioremediation rarely restores an environment completely. Often the residual contamination left after treatment is strongly sorbed and not available to microorganisms for degradation. Over a long period of time (years), these residuals can be slowly released. There is little research concerning the fate and potential toxicity of such released residuals, and therefore there is both public and regulatory concern about the importance of residual contamination.

Although it is often not thought of as bioremediation, domestic sewage waste has been treated biologically for many years with resounding success. In application of bioremediation to other environmental problems, it must be kept in mind that biodegradation is dependent on the pollutant structure and bioavailability. Therefore, application of bioremediation to other pollutants depends on the type of pollutant or pollutant mixtures present and the type of microorganisms present. The first successful application of bioremediation outside sewage treatment was the cleanup of oil spills, and success in this area is now well documented (see Case Study 3). In the past few

CASE STUDY 3

Exxon *Valdez*

In March 1989, 33,000 tons of crude oil were spilled from the Exxon *Valdez* in the Prince William Sound, Alaska. This oil was subsequently spread by a storm and coated the shores of the islands in the Sound. Conventional cleanup, primarily by physical methods, did not remove all of the oil on the beaches, particularly under rocks and in the beach sediments. Exxon reached a cooperative agreement with the Environmental Protection Agency (EPA) in late May 1989 to test bioremediation as a cleanup strategy.

The approach followed was to capitalize on existing conditions as much as possible. For example, for oil-degrading microorganisms, scientists realized that the beaches most likely contained indigenous organisms that were already adapted to the cold climate. In fact, preliminary studies showed that, indeed, the polluted beaches contained such organisms as well as a plentiful carbon source (spilled oil) and sufficient oxygen. In fact, intrinsic biodegradation was already occurring in the area (Button, 1992). Further studies showed that intrinsic degradation rates were limited by availability of nutrients such as nitrogen, phosphorus, and other trace elements. Time was of the essence in treating the contaminated sites because the relatively temperate Alaskan season was rapidly advancing. Therefore, it was decided to attempt to enhance the intrinsic rates of biodegradation by amendment of the contaminated areas with nitrogen and phosphorus.

In early June 1989, field demonstration work was started. Several different fertilizer nutrient formulations and application procedures were tested. One problem encountered was the tidal action, which tended to quickly wash away added nutrients. Therefore a liquid oleophilic fertilizer (Inipol EAP-22) that adhered to the oil-covered surfaces and a slow-release water-soluble fertilizer (Customblen) were tested as nutrient sources. Within about 2 weeks after application of the fertilizers, there was a visible decrease in the amount of oil on rock surfaces treated with Inipol EAP-22. Subsequent independent scientific studies of the effectiveness of bioremediation in Prince William Sound have concluded that bioremediation enhanced removal from three- to eightfold over the intrinsic rate of biodegradation without any adverse effects on the environment. Bioremediation in Prince William Sound was considered a success because the rapid removal of the petroleum contamination prevented further spread of the petroleum to other uncontaminated areas and restored the contaminated areas. This well-documented and highly visible case study has helped gain attention for the potential of bioremediation. It should be noted that while the bioremediation was considered successful; today, more than 10 years later, weathered oil can still be found at dozens of sites in the Prince William Sound even hundreds of miles from the spill site. Thus, bioremediation is not a perfect solution, and the question discussed in Chapter 16.1 remains; "How clean should clean be?"

years, many new bioremediation technologies have emerged that are being used to address other types of pollutants (Table 16.3). In fact, every other year, beginning in 1991, a meeting entitled "International *In Situ* and On-Site Bioreclamation Symposium" has been held so that the latest technologies in bioremediation can be presented, discussed, and published.

Several key factors are critical to successful application of bioremediation: environmental conditions, contaminant and nutrient availability, and the presence of degrading microorganisms. If biodegradation does not occur, the first thing that must be done is to isolate the factor limiting bioremediation, and this can sometimes be a very difficult task. Initial laboratory tests using soil or water from a polluted site can usually determine whether degrading microorganisms are present and whether there is an obvious environmental factor that limits biodegradation, for example, extremely low or high pH or lack of nitrogen and/or phosphorus. However, sometimes the limiting factor is not easy to identify. Often pollutants are present as mixtures and one component of the pollutant mixture can have toxic effects on the growth and activity of degrading microorganisms. Low bioavailability due to sorption and aging is another factor that can limit bioremediation and can be difficult to evaluate in the environment.

Actual application of bioremediation is still limited in practice but is rapidly gaining in popularity. Most of the developed bioremediation technologies are based on two standard practices: addition of oxygen and addition of other nutrients (Norris, 1994).

16.7.1 Addition of Oxygen or Other Gases

One of the most common limiting factors in bioremediation is availability of oxygen. Oxygen is an element required for aerobic biodegradation. In addition, oxygen has low solubility in water and a low rate of diffusion (movement) through both air and water. The combination of these three factors makes it easy to understand that inadequate oxygen supplies will slow bioremediation. Several technologies have been developed to overcome a lack of oxygen. A typical bioremediation system used to treat a contaminated aquifer as well as the contaminated zone above the water table is shown in Fig. 16.28a. This system contains a series of injection wells or galleries and a series of recovery wells that comprise a two-pronged approach to bioremediation. First, the recovery wells remove contaminated groundwater, which is treated above ground, in this case using a **bioreactor** containing microorganisms that are acclimated to the contaminant. This would be considered *ex situ* treatment. Followng bioreactor treatment, the clean water is supplied with oxygen and nutrients, and then it is reinjected into the site. The reinjected water provides oxygen and nutrients to stimulate *in situ* biodegradation. In addition, the reinjected water flushes the vadose zone to aid in removal of the contaminant for above-ground bioreactor treatment. This remediation scheme is a very good example of a combination of physical, chemical, and biological treatments being used to maximize the effectiveness of the remediation treatment.

Bioventing is a technique used to add oxygen directly to a site of contamination in the vadose zone (unsaturated zone). Bioventing is a merging of soil vapor extraction technology and bioremediation. The bioventing zone is highlighted in red in Fig. 16.28b and includes the vadose zone and contaminated regions just below the water table. As shown in this figure, a series of wells have been constructed around the zone of contamination. To initiate bioventing, a vacuum is drawn on these wells to force accelerated air movement through the contamination zone. This effectively increases the supply of oxygen throughout the site, and thus the rate of contaminant biodegradation. In some cases, depending on the type of pollutant in the site, pollutant volatility becomes an issue, e.g., in gasoline spills. In this case, some of the pollutants will be removed as air is forced through this system. This contaminated air can also be treated biologically by passing the air through aboveground soil beds in a process called **biofiltration** as shown in Fig. 16.29 (Jutras *et al.*, 1997).

In contrast, **air sparging** is used to add oxygen to the saturated zone (Fig. 16.28c). In this process, an air sparger well is used to inject air under pressure below the water table. The injected air displaces water in the soil matrix, creating a temporary air-filled porosity. This causes oxygen levels to increase, resulting in enhanced biodegradation rates. In addition, volatile organics will volatilize into the airstream and be removed by a vapor extraction well.

Methane is another gas that can be added with oxygen in extracted groundwater and reinjected into the saturated zone. Methane is used specifically to stimulate methanotrophic activity and cometabolic degradation of chlorinated solvents. As described in Chapter 14.2.3.3, methanotrophic organisms produce the enzyme methane monooxygenase to degrade methane, and this enzyme also cometabolically degrades several chlorinated solvents. Cometabolic degradation of chlorinated solvents is presently being tested in field trials to determine the usefulness of this technology.

16.7.2 Nutrient Addition

Perhaps the second most common bioremediation treatment is the addition of nutrients, in particular nitrogen and phosphorus. Many contaminated sites con-

TABLE 16.3 Current Status of Bioremediation

Chemical class	Frequency of occurrence	Status of bioremediation	Evidence of future success	Limitations
Hydrocarbons and derivatives				
Gasoline, fuel oil	Very frequent	Established		Forms nonaqueous phase liquid
PAHs	Common	Emerging	Aerobically biodegradable under a narrow range of conditions	Sorbs strongly to subsurface soilds
Creosote	Infrequent	Emerging	Readily biodegradable under aerobic conditions	Sorbs strongly to subsurface soilds; forms nonaqueous phase liquid
Alcohols, ketones, esters	Common	Established		
Ethers	Common	Emerging	Biodegradable under a narrow range of conditions using aerobic or nitrate-reducing microbes	
Halogenated aliphatics				
Highly chlorinated	Very frequent	Emerging	Cometabolized by anaerobic microbes; cometabolized by aerobes in special cases	Forms nonaqueous phase liquid
Less chlorinated	Very frequent	Emerging	Aerobically biodegradable under a narrow range of conditions; cometabolized by anaerobic microbes	Forms nonaqueous phase liquid
Halogenated aromatics				
Highly chlorinated	Common	Emerging	Aerobically biodegradable under a narrow range of conditions; cometabolized by anaerobic microbes	Sorbs strongly to subsurface solids; forms nonaqueous phase either liquid or solid
Less chlorinated	Common	Emerging	Readily biodegradable under aerobic conditions	Forms nonaqueous phase either liquid or solid
Polycholorinated biphenyls				
Highly chlorinated	Infrequent	Emerging	Cometabolized by anaerobic microbes	Sorbs strongly to subsurface solids
Less chlorinated	Infrequent	Emerging	Aerobically biodegradable under a narrow range of conditions	Sorbs strongly to subsurface solids
Nitroaroamatics	Common	Emerging	Aerobically biodegradable; converted to innocuous volatile organic acids under anaerobic conditions	
Metals (Cr, Cu, Ni, Pb, Hg, Cd, Zn, etc.)	Common	Possible (see Chapter 17)	Solubility and reactivity can be changed by a variety of microbial processes	Availability highly variable and controlled by solution and solid-phase chemistry

Adapted from National Research Council (1993).

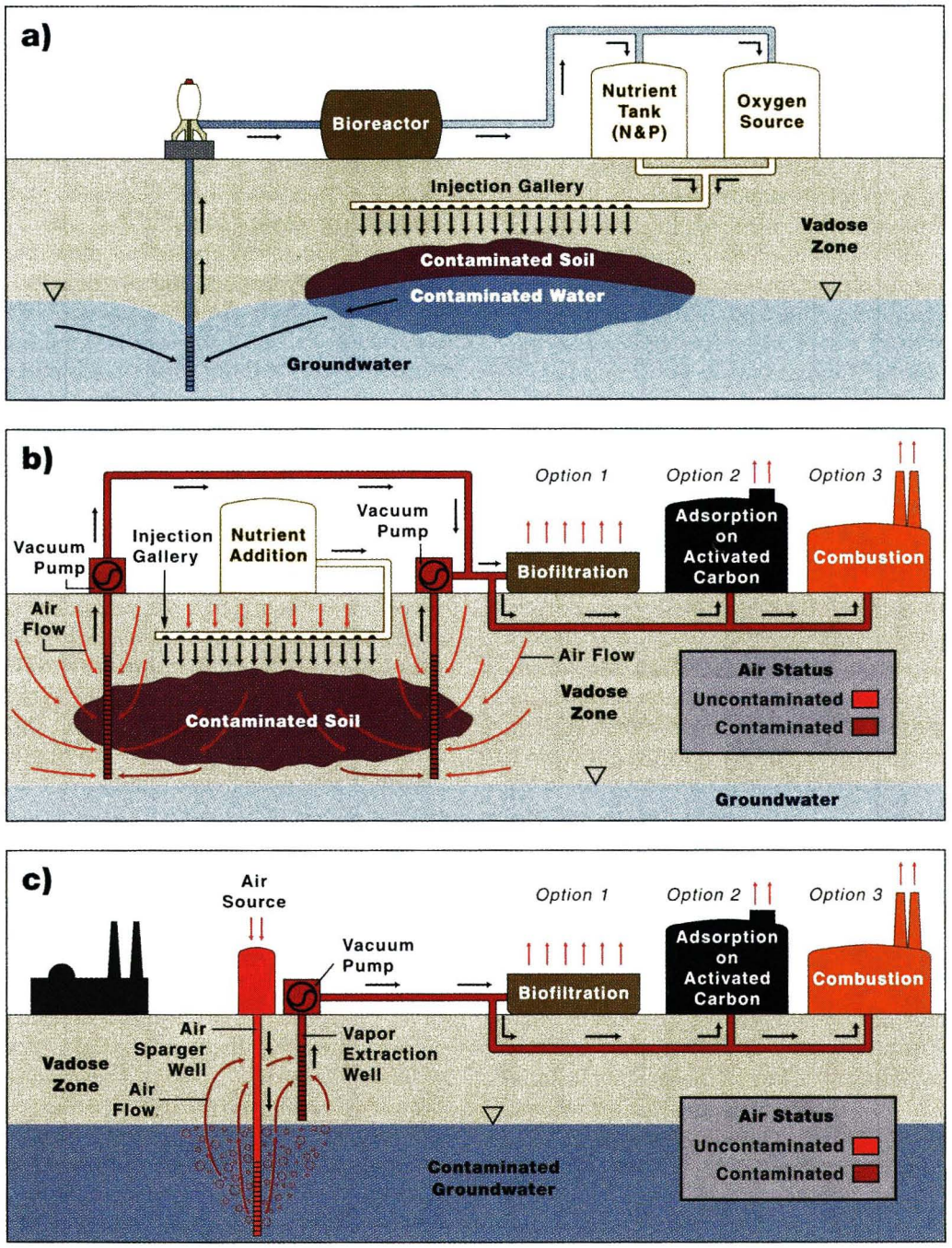
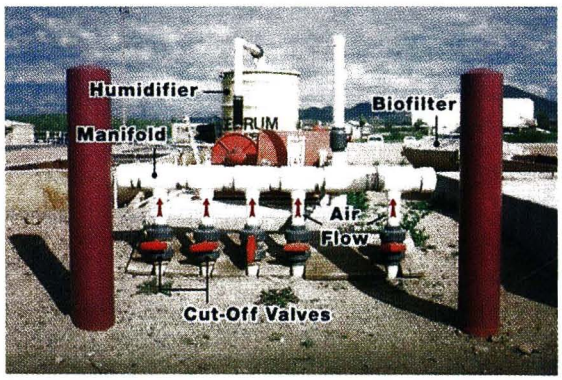
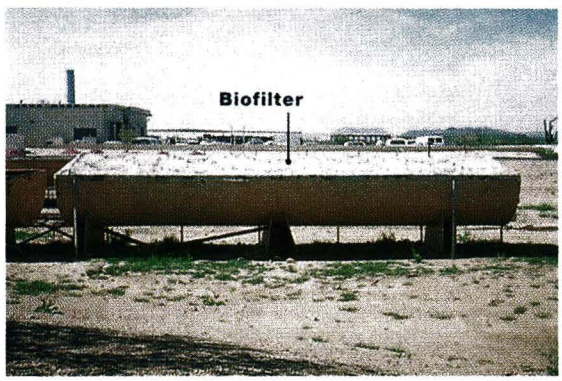


FIGURE 16.28 (a) *In situ* bioremediation in the vadose zone and groundwater. Nutrients and oxygen are pumped into the contaminated area to promote *in situ* processes. This figure also shows *ex situ* treatment. *Ex situ* treatment is for water pumped to the surface and uses an aboveground bioreactor, as shown, or other methods, e.g., air stripping, activated carbon, oil-water separation, or oxidation. An injection well returns treated water to the aquifer. (b) Bioventing and biofiltration in the vadose zone. Air drawn through the contaminated site (bioventing) stimulates *in situ* aerobic degradation. Volatile contaminants removed with the air are treated in a biofilter, by adsorption on activated carbon, or by combustion. (c) Bioremediation in the groundwater by air sparging. Air pumped into the contaminated site stimulates aerobic degradation in the saturated zone. Volatile contaminants brought to the surface are treated by biofiltration, activated carbon, or combustion. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)



a) Soil vapor extraction unit



b) Side view of biofilter

FIGURE 16.29 Bioremediation of petroleum vapors from a leaking underground storage tank. (a) Red arrows denote the direction of airflow out of the ground and through the manifold, which controls the airflow. The air then passes through the humidifier, a tank containing water, and into the biofilter. The large red vertical cylinders serve as protective barriers. (b) Air flows into the biofilter through a pipe running lengthwise along the bottom of the filter and into the soil via perforations in the pipe. (From *Pollution Science* © 1996, Academic Press, San Diego, CA.)

tain organic wastes that are rich in carbon but contain minimal amounts of nitrogen and phosphorus. Nutrient addition is illustrated in the bioremediation schemes shown in Fig. 16.28a and b. Injection of nutrient solutions takes place from an above-ground batch feed system. The goal of nutrient injection is to optimize the ratio of carbon, nitrogen, and phosphorus (C:N:P) in the site to approximately 100:10:1. However, sorption of added nutrients can make it difficult to achieve the optimal ratio accurately.

16.7.3 Stimulation of Anaerobic Degradation Using Alternative Electron Acceptors

Until recently, anaerobic degradation of many organic compounds was not even considered feasible (Grbíc-Galíc, 1990). Now it is being proposed as an alternative bioremediation strategy even though aerobic degradation is generally considered a much more rapid process. This is because it is difficult to establish and maintain aerobic conditions in some groundwater sites. Several alternative electron acceptors have been proposed for use in anaerobic degradation, including nitrate, sulfate, iron (Fe^{3+}), and carbon dioxide. There has even been a limited number of field trials using nitrate that show promise for this approach. This is a relatively new area in bioremediation that will undoubtedly receive increased attention in the next few years.

16.7.4 Addition of Surfactants

Surfactant addition has been proposed as a technique for increasing the bioavailability and hence biodegradation of contaminants (see Section 16.4.2). Surfactants can be synthesized chemically and are also produced by many microorganisms, in which case they are called biosurfactants. Surfactants work similarly to industrial and household detergents that effectively remove oily residues from machinery, clothing, or dishes. As shown in the cartoon in Fig. 16.8, individual contaminant molecules can be "solubilized" inside surfactant micelles. These micelles range from 5 to 10 nm in diameter. Alternatively, surfactant molecules can coat oil droplets and emulsify them into solution. In addition, biosurfactants seem to enhance the ability of microbes to stick to oil droplets. In laboratory tests, synthetic surfactants and biosurfactants can be used to increase the apparent aqueous solubility of organic contaminants. However, field tests have been attempted only with synthetic surfactants and results have been mixed (Miller, 1995).

16.7.5 Addition of Microorganisms or DNA

If appropriate biodegrading microorganisms are not present in soil or if microbial populations have been reduced because of contaminant toxicity, specific microorganisms can be added as "introduced organisms" to enhance the existing populations. This process is known as **bioaugmentation.** Scientists are now capable of creating "superbugs," organisms that can degrade pollutants at extremely rapid rates. Such organisms can be developed through successive adaptations under laboratory condition, or can be **genetically engineered.** In terms of biodegradation, these superbugs are far superior to organisms found in the environment. The problem is that introduction of a microorganism to a contaminated site may fail for two reasons. First, the introduced microbe often cannot establish a niche in the environment. In fact, these introduced organisms often do not survive in a new

environment beyond a few weeks. Second, there are difficulties in delivering the introduced organisms to the site of contamination, because microorganisms, like contaminants, can be strongly sorbed by solid surfaces. Currently, very little is known about microbial transport and establishment of environmental niches. These are areas of active research, and in the next few years scientists may gain a further understanding of microbial behavior in soil ecosystems. However, until we discover how to successfully deliver and establish introduced microorganisms, their addition to contaminated sites will not be a viable bioremediation option.

One way to take advantage of the superbugs that have been developed is to use them in bioreactor systems under controlled conditions. Extremely efficient biodegradation rates can be achieved in bioreactors that are used in aboveground treatment systems.

A second bioaugmentation strategy is to add specific genes that can confer a specific degradation capability to indigenous microbial populations. The addition of degradative genes relies on the delivery and uptake of the genetic material by indigenous microbes. There are two approaches that can be taken in delivery of genes. The first is to use microbial cells to deliver the DNA via conjugation. The second is to add "naked" DNA to the soil to allow uptake via transformation. This second approach may reduce the difficulty of delivery since DNA alone is much smaller than a whole cell. However, little is known as yet about these two approaches. As discussed in the Chapter 3 Case Study, Di Giovanni *et al.* (1996) demonstrated that gene transfer can occur in soil resulting in 2,4-D degradation activity. However, whether such transfer is common, and conditions that are conducive or inhibitory to such transfer are not yet defined.

QUESTIONS AND PROBLEMS

1. Why is there concern about the presence of organic contaminants in the environment?
2. Describe the different factors that can limit biodegradation of organic contaminants in the environment.
3. Draw and name an aliphatic, alicyclic, and aromatic structure, each with 6 carbons.
4. Outline the biodegradation pathway for each of the structures that you just drew under aerobic conditions.
5. Why are aerobic conditions usually preferred for biodegradation of organic contaminants? Under what conditions might anaerobic biodegradation be preferred?
6. Compare the advantages and disadvantages of intrinsic, *ex situ*, and *in situ* bioremediation.
7. You have been hired to bioremediate a site in which the groundwater is contaminated with petroleum. Groundwater samples have a strong sulfide smell and gas chromatographic analysis of the samples show negligible biodegradation of the petroleum has occurred. What is your recommendation?
8. Kleen Co. is in charge of a site in Nevada that was used for pesticide preparation. As a result of years of operation, the groundwater below this site has elevated levels of pesticides (up to 20 mg/l). Your initial investigation shows that 1) the pesticide-containing plume is neither growing nor shrinking in size, 2) there are pesticide degraders in the plume, 3) and the dissolved oxygen levels in the plume range from 2 to 4 mg/l. This site is not being used presently, and the groundwater is not used for drinking water purposes. What is your best recommendation based on these site characteristics and on your knowledge of cost of remediation?

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