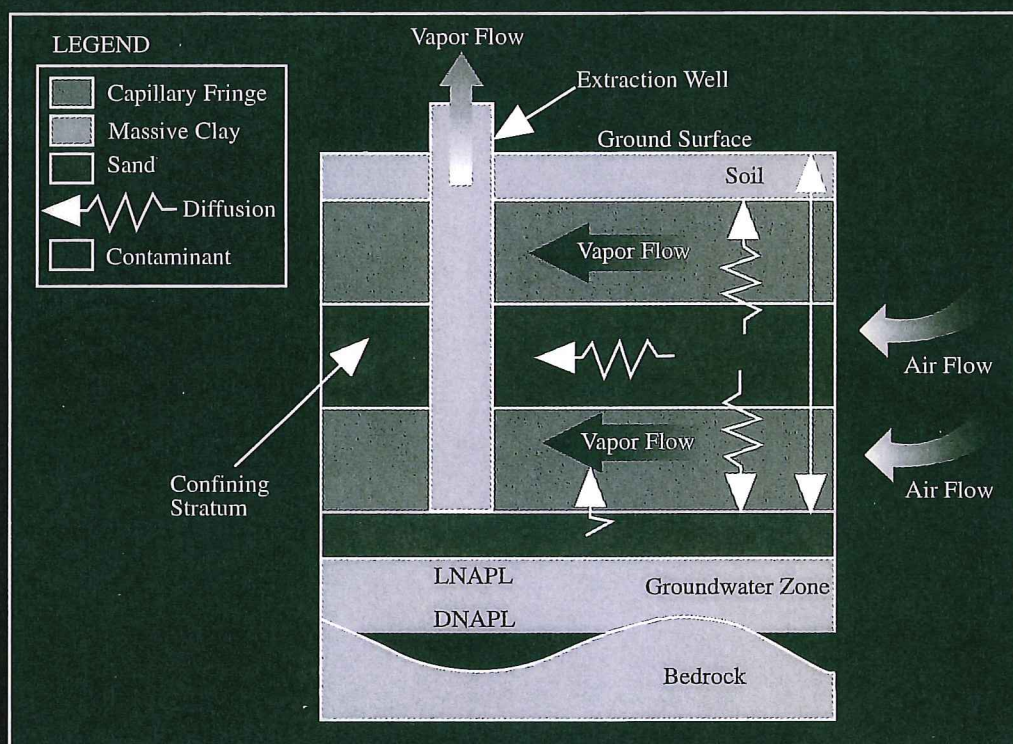


HAZARDOUS WASTE SITE REMEDIATION

Source Control



Domenic Grasso

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BIOREMEDIATION

Instructional objectives:

- Discuss basic microbial processes occurring during bioremediation
- Quantify mass transport effects in bioremediation
- Discuss kinetics of bioremediation
- Identify optimum conditions for bioremediation
- Determine feasibility of alternative bioremediation methods
- Develop a systematic approach for using bioremediation

1. MICROBIAL PROCESSES OCCURRING DURING BIOREMEDIATION

1.1. Overview

Bioremediation is defined as the use of microorganisms to mediate the transformation of hazardous chemicals to less toxic and environmentally acceptable compounds.

Bioremediation is a process that utilizes microbial organisms, or their metabolic products to convert complex substances (substrates) to simpler products, typically reducing their toxicity to the environment. The process breaks down the substrate and utilizes the chemical energy liberated to drive the thermodynamically non-spontaneous process, such as synthesis of the cell components. The process converts complex substances, using them as an energy source or carbon source, to simpler compounds. Bioremediation typically effects in an increase in the redox potential of the substrate. When an organic compound is used as a carbon source, and oxygen is used as the terminal electron acceptor (aerobic processes), carbon dioxide is typically liberated as a byproduct of the process.

Biological treatment can be an efficient and cost effective way to remove hazardous substances from:

- Liquid
- Vapor
- Solid Phases

A myriad of bioremediation technologies have been explored, ranging from attempts to utilize bacterial cultures typically found in the environment to sophisticated genetic engineering applications that produce tailor made organisms capable of degrading specific compounds. Among the innovative or emerging methods are:

- Plasmid-assisted molecular breeding
- Gene-engineering
- Enhancement of indigenous microorganisms.

Xenobiotic compounds are typically somewhat recalcitrant to biodegradation. Xenobiotic compounds are chemical structures that involve novel chemical groups or substitutes found rarely in nature. Hence, these compounds undergo slow biological transformations, tending to persist and accumulate in the environment.

The fate and transport of xenobiotic compounds introduced into the environment are impacted by:

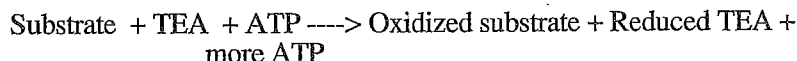
- Sorption
- Coagulation
- Precipitation
- Biotransformation
- Photolysis
- Hydrolysis
- Oxidation / Reduction

Naturally existing microorganisms adapt to degrade xenobiotic compounds either by:

- Utilizing existing catabolic enzymes, or
- Acquiring new metabolic pathways

Bioremediation can be a feasible remedial technology if the microorganisms are capable of acquiring new metabolic pathways and synthesizing appropriate enzymes to achieve the desired degradation within a reasonable period of time.

The generalized chemical reactions for a microbial growth can be depicted as follows:



TEA = Terminal Electron Acceptor

ATP = Adenosine Triphosphate (Energy currency of the cells)

1.2. Microorganisms

Microorganisms fall under the kingdom of Protists. Because Protists consist of the fundamental and earlier forms of life, they have simple metabolic and physiological requirements. The Protists are further divided into two categories, Eucaryotes and Procaryotes.

Procaryotes - consist of organisms like bacteria, blue green algae and a few others having rather "simple" cell structures. The typical length of procaryotic cells is 5 μm and weighs around 10 pico grams.

Bacteria, classified as prokaryotic organisms, generally reproduce by the process of binary fission, in which a cell enlarges due to new cell-wall growth and then divides, producing two daughter cells of equal size.

Eucaryotes - consist of protozoa, algae, fungi etc. having larger and more advanced cell structure.

Chemical formulas have often been used to represent the proportions of various elements in a bacterial cell. The most commonly used formula is $C_6H_7NO_2$. Typical bacteria cell composition consists of 50% carbon, 20% oxygen, 10-15% nitrogen 5% hydrogen and a small fraction of other elements like phosphorus and sulfur. Since carbon constitutes the major elemental fraction of a cell, carbon sources are important factors for cell growth. Carbon is required for the synthesis of amino acids, enzymes, nucleic acids, vitamins etc. Cells vary in their ability to synthesize materials they require to thrive and reproduce.

1.3. Classification

All microorganisms require a carbon source for cell growth and an energy source to sustain the metabolic functions required for growth. The nature of microbial metabolism is diverse and therefore the source of carbon and energy used by the microbes define their classification. The following definitions illustrate some of the various categories.

Microbe classifications are based on:

C source:

Heterotrophic - use organic compounds (e.g. glucose) as a carbon source

Autotrophic - use inorganic carbon compounds (e.g. carbon dioxide) as a carbon source

Energy source:

Chemotrophic - chemical reactions provide the energy for growth. If inorganic compounds provide the energy they are called Lithotrophic; if the source of energy is from an organic compound, they are known as Organotrophic

Phototrophic - use sunlight or other form of light as the energy source

TEA:

Aerobes - oxygen is the terminal electron acceptor

Anaerobes - a compound other than oxygen is the terminal electron acceptor

Facultative - can degrade both in absence or presence of oxygen, though the growth yield is normally higher in presence of oxygen

These terms are often used in combination with each other.

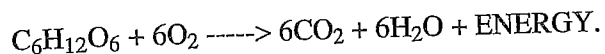
Example: A human is a Hetero-organotroph, meaning he/she uses organic carbon for cell growth and organic chemicals for the energy required for growth. Similarly plants are auto-phototrophs and require an inorganic carbon source, carbon dioxide, and sunlight as energy sources. Plants are auto-phototrophs in the presence of sunlight as explained above; in the absence of sunlight, however, plants may be classified as Hetero-organotrophs.

Sometimes terms like "obligate anaerobe" are used for classification, which indicates that the microorganism can not grow in the presence of air. Therefore, the species of this microbe would be more abundant in the oxygen depleted zones.

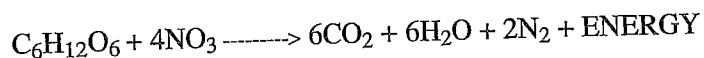
Aerobic microorganisms are often used to treat water streams and other media exposed to air. Aerobic treatment can also be used to bioremediate subsurface environments as long as an oxygen source is provided. Anaerobic organisms, while useful for treatment of organic sludges and slurry organic waste, may not be advantageous for subsurface bioremediation in view of their low growth rate and slow response in subsurface environments. Applicability of anaerobic treatment in subsurface environments is currently under research.

Having knowledge of growth characteristics of these organisms assists in predicting their habitats and feasibility for use in environmental remediations; likewise, knowledge of environmental conditions assists in predicting the type of species and degradation pathways likely to be present.

For example, when glucose is degraded aerobically, it can be represented by the equation given below:



Similarly, anaerobic degradation of glucose in the presence of nitrate may be written as:



In view of the lower position of oxygen in the electron tower, as compared to nitrate, more energy is liberated under aerobic oxidation of glucose. i.e. when oxygen is used as the terminal electron acceptor as compared to anaerobic oxidation with nitrate as the TEA. Due to the lower energy liberated in anaerobic oxidation, the rate of cell synthesis is lower in anaerobic environments as compared to aerobic ones.

In addition to carbon and energy sources, microorganisms also require nutrients for growth and for metabolic activities.

1.4. Definitions

Catabolic Process - the biochemical process involved in the breakdown of complex compounds to simpler molecules, usually leading to the production of energy; e.g. Glycolysis breaks down glucose to pyruvate, yielding 2 moles of ATP for every mole of glucose.

Anabolic Process - the biochemical process involved in the building up of cell components and other macro molecules, usually requiring energy; e.g. protein synthesis (carried out in ribosomes) synthesizes proteins from amino acids.

Oligotrophic - describing a body of water in which nutrients are in low supply

Xenobiotic - a completely synthetic chemical compound not naturally occurring on earth

Macronutrients - elements needed in major quantities - C, K, Mg, Ca, H, N, O, S, P, Na, Cl

Micronutrients - elements required in small quantities - Co, Ni, Fe, Zn, Mo, Wb, Se

Neutralism - when there is no competition for substrate or any interaction between two species in a given microbial community

Mutualism - when two species are mutually benefitted by each other in a microbial community. For example, in a pond, the bacteria produce carbon dioxide from a carbon source only in the presence of oxygen. The carbon dioxide helps algae synthesize cell mass in the presence of sunlight, releasing the needed oxygen as a by-product

Antagonistic - when the growth of one species hampers or inhibits the growth of another species. This can occur in several ways: competition for resources, amensalism (release of chemicals having deleterious effects on other organisms), and predation

Comensalism Metabolism- when one species is benefited by the growth of another while the other is unaffected, e.g.. growth factor secretion, cellulose degradation resulting in free monomers that may act as substrates for others

Gratuitous - when enzymes for biodegradation are induced due to similarity of biodegradation between compounds, e.g.. Cresol is degraded by the microbes degrading Toluene due to the similarity in the structure of the two compounds

Cometabolism - transformation of a non-growth substrate in the presence of another transformable compound, e.g.. TCE is degraded in the presence of methane and oxygen but remains unaffected otherwise

Further information on the basic mechanism of biodegradation is provided in the appendix.

2. MASS TRANSPORT ISSUES DURING BIOREMEDIATION

Bioremediation processes can be used to treat contaminants present in the liquid phase, vapor phase and/or solid phase. The microorganisms needed for bioremediation can either be present as a suspension in the liquid phase or attached to a solid surface. Mass transport in bioremediation refers to the transport of the reactant (contaminant, nutrients) to the microorganisms, which is necessary for biodegradation of the contaminant to occur.

Biodegradation occurs in the liquid phase when the microorganisms are suspended in water and assimilate chemical compounds dissolved in the liquid phase. The chemical compounds diffuse to the cell wall of the microorganisms and are then eventually transported into the cell for biological growth and energy. The potential for microorganisms to consume or biodegrade a chemical compound is directly dependent on the chemicals ability to dissolve in water (solubility) and diffuse through the cell wall (diffusion coefficient in water).

If a solid phase is present, such as soil, debris, etc., the microorganisms may attach to the solid phase, forming a biological film on the solid surface. In this case, the chemical compound diffuses through the biological film covering a solid surface, and then adsorbs on the cell walls. It then undergoes two types of transport:

- Diffusion through the cell wall into the cell, wherein it is consumed (biodegraded) for growth and energy
- Diffusion to the walls of other adjacent cells, i.e. diffusion through the biofilm, and then subsequent biodegradation by the other cells constituting the biofilm

The net result is the gradual diffusion of the compound through the biofilm and simultaneous biodegradation by the microorganisms.

As a result of this biodegradation and consequent growth of the cell population, the biofilm thickness increases, which increases the resistance to diffusion. Eventually, a biofilm thickness is achieved, where the microbial cells attached to the solid surface on the inner surface of the biofilm are able to completely consume the amount of the chemical compound reaching the cells through the diffusion process; hence, no additional compound enters the solid phase. This results in a balance between the amount of the compound diffusing into the biofilm and the amount biodegrading in the biofilm (steady-state).

The actual thickness of a biofilm depends on several factors:

- The rate of diffusion of the chemical compound to the biofilm (external diffusion) and the rate of diffusion through the biofilm (internal diffusion)

- The rate at which the cells are removed from the biofilm by death, decay and/or predation by other species and also by hydrodynamic shear.

2.1. Mass Transport in The Liquid Phase

There are two mechanisms by which a chemical can be transported through a liquid phase:

- Diffusion due to molecular motion, known as "Fickian Diffusion"
- Transport by eddies formed in the liquid phase due to local turbulence caused by mixing, known as "Eddy Diffusion"

Diffusion can be characterized by a diffusion equation defined as follows:

$$R = (D_m + D_e) \left(\frac{dC}{dx} \right) \quad (1)$$

where:

- R = the rate of transport of contaminant or chemical per unit area per unit time
- D_m = the diffusion coefficient due to molecular motion (Fickian diffusion)
- D_e = the diffusion coefficient due to movement of eddies (Eddy diffusion)
- dC/dx = the concentration gradient in the liquid phase

Fickian diffusion coefficients, D_m , are measured experimentally for a given chemical and liquid, and tabulated in handbooks. They can also be estimated, if not measured experimentally.

Eddy diffusion coefficients depend on the type of system, amount of mixing, liquid phase properties, etc.

Since eddy diffusion coefficients are not known generally, the term $(D_m + D_e)(dC/dx)$ in equation (1) is simplified as follows:

$$(D_m + D_e) \left(\frac{dC}{dx} \right) = k(C_1 - C_2) \quad (2)$$

where:

- k = the "mass transfer coefficient"
- C_1, C_2 = concentrations in the liquid phase

The mass transfer coefficient, k, is calculated from empirical equations based on experimentally determined correlations.

2.2. Mass Transport in Biofilms

When a solid phase is present in the process, cell attachment to the solid surface and subsequent cell growth results in the formation of a biofilm on the solid surface. In this case, biodegradation occurs in this biofilm through the process of simultaneous diffusion and biodegradation.

Fixed bed processes behave similar to plug-flow reactors, where the liquid phase flows through the system without mixing. Fluidized bed processes behave like well-mixed reactors where the liquid phase is continuously mixed by the fluidized beads.

The mass transport in biofilm processes can be quantified similarly to the liquid phase processes. The rate of transport through the biofilm can be written as follows:

$$R = D_f \left(\frac{dC_f}{dz} \right)$$

where:

- R = rate of transport per unit area per unit time through the biofilm
- D_f = the Fickian diffusion coefficient of the chemical in the biofilm
- dC_f/dz = the concentration gradient of the chemical in the biofilm

Note that only the Fickian diffusion coefficient is used here since the biofilm is stagnant, i.e., it is attached to the solid surface.

Furthermore, at steady-state, when the rate of transport of chemicals through the biofilm is balanced by its rate of degradation, we can write:

$$R = \text{rate of chemical biodegradation in the biofilm}$$

The rate of biodegradation, presented later, will be quantified using equations of chemical kinetics.

2.3. Mass Transport in The Solid Phase

Bioremediation in solid phase such as in soils and sediments, involves the transport of the contaminant from the solid phase to the microorganisms. The contaminant desorbs from the solid surface. The rate of desorption depends on the type of chemical and the nature of its interaction with the solid phase. Usually, the solid phase contains pores, and the contaminant diffuses out of the pores into the liquid or gas phase. The microorganisms can be present as a suspension in the liquid phase within saturated pores or attached to the solid phase either inside or outside the pores.

In principle, diffusion rates can be quantified by equation where there is no eddy diffusion, i.e., $D_e=0$, and the Fickian diffusion coefficient, D_m , is replaced by a diffusion coefficient representing diffusion in the pores of the solid phase. However, diffusion in the pores of a solid phase, such as soil or sediment, has several complexities. Some of these are:

- The diffusion coefficient is confined to the pores of the solid phase, since the contaminant does not diffuse through the solid phase itself—therefore the path is tortuous and unpredictable due to heterogeneous distributions of pore sites
- The effective diffusion coefficient is not independent of concentration
- Adsorption and desorption on the solid phase surface occurs
- The diffusion coefficient depends on the properties of the solid phase (soil) such as mineral composition, organic matter content, bulk density, water content, and temperature

3. BIOREMEDIATION PROCESS APPLICATIONS

Bioremediation processes can be classified into three main types:

- Above-ground treatment of contaminated soils
 - Biopiles/landfarming/composting

- Slurry reactors
- Above-ground treatment of contaminated water
 - Bioreactors
- *In situ* treatment
 - Saturated zone
 - Unsaturated zone

3.1. Aboveground Treatment of Soils

Aboveground or ex-situ treatment of contaminated soils can be achieved using biopiles, landfarming, or composting.

3.1.1. Biopiles

A biopile is created by spreading contaminated soil on a lined treatment bed. Air and/or nutrients dissolved in water are passed through the soil pile to enhance biodegradation of the contaminants present in the soil.

There are two types of biopiles:

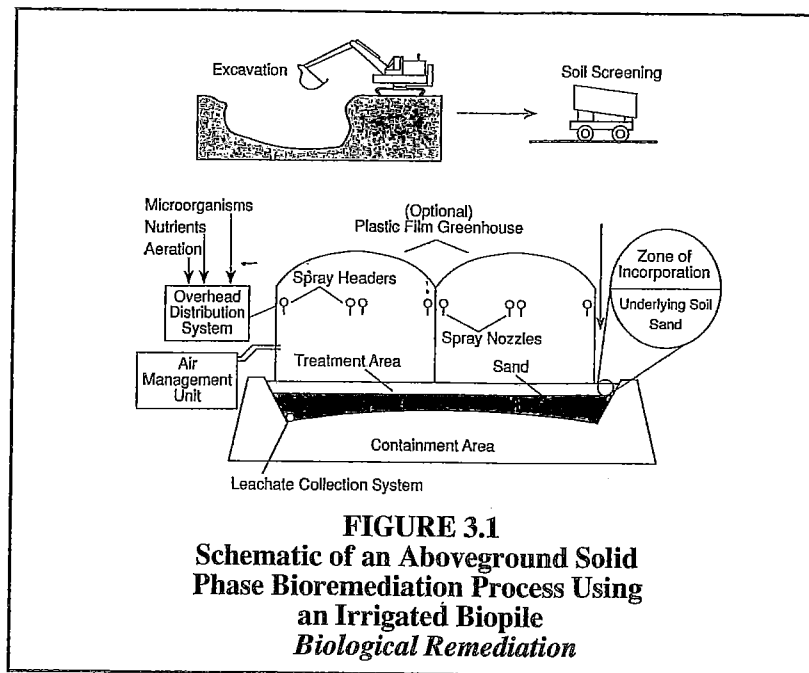
- Oxygen and nutrients are delivered to the bacteria by water movement through the soil pile. The water is added by an irrigation system and the bed is equipped with a drainage collection system. The drainage from the soil pile, or leachate, is treated in a liquid phase bioreactor and recirculated through the soil pile. The soil pile may be totally enclosed if volatile- emissions control is necessary. Figure 3.1 shows aboveground solid-phase bioremediation using an irrigated biopile
- Nutrients are mixed with the soil when the soil pile is created and oxygen is delivered by pumping air through the soil. The air is delivered by pipes placed at the bottom and middle of the soil pile. A plastic liner is placed on top of the pile to prevent rainwater from permeating into the pile

3.1.2. Landfarming

Landfarming is the practice of spreading contaminated soils or wastes on the surface of the ground to facilitate natural microbial degradation of contaminants. Landfarming was the first method used for bioremediation of soils and sludges and has been successfully applied by the petroleum industry for disposal of petroleum-refinery wastes. Landfarming is typically used in areas where air emission would not pose unacceptable health risks and would not require collection.

3.1.3. Composting

Composting is an established technology for management of municipal wastewater treatment sludges and is an emerging technology for treatment of hazardous wastes. Biodegradation of organic compounds occurs within a compost matrix consisting of contaminated soils and/or sludges mixed with organic carbon sources and bulking agents such as straw, bark, wood chips, sawdust, etc. The compost piles, which are three to six feet in height, are placed on lined treatment beds and the microbial degradation is enhanced by maintaining optimum temperature, moisture content, oxygen, and nutrient levels within the pile.



3.1.4. Slurry reactors

In a slurry phase reactor, soil or sludge and nutrient-containing water are combined to form an aqueous slurry wherein biodegradation of the contaminants occurs. Mixing must be sufficient to keep the solids in suspension and oxygen must be supplied throughout the slurry-phase to promote aerobic microbial activity.

Slurry-phase biotreatment may be performed either in bioreactor vessels or in lined lagoons.

3.2. Aboveground Treatment of Water

Aboveground treatment of contaminated water can be achieved in either suspended growth reactors or fixed film reactors. In suspended growth reactors the microorganisms are kept suspended in the aqueous phase. In fixed film reactors the microorganisms are grown in biofilms covering solid surfaces, and the contaminated water is circulated to maximize contact with the biofilm.

3.2.1. Suspended Growth Reactors

Suspended growth bioreactors are of two types:

- Completely-mixed systems, in which the microbial cells are suspended in the liquid phase, and the suspension is stirred to obtain a mixed state between the cells and the liquid phase
- Plug flow systems, in which the microbial cell suspension flows without axial dispersion in a tubular reactor

If oxygen is required for the cells, the water is either saturated by oxygen before it enters the reactor, or air containing oxygen is introduced to the liquid phase (air sparging). The chemical

compounds and nutrients, dissolved in the water, diffuse into the cells and are biodegraded into carbon dioxide or transformed into metabolite products.

The well-mixed liquid phase process can be operated in either a "batch" or "continuous" mode. In the batch operating mode, the liquid phase and microbial cells are charged into the reactor, mixed, and when the compounds present in the liquid phase have been biodegraded, to target concentrations, the reactor contents are discharged. As oxygen is slightly soluble in the culture medium, a continuous oxygen supply is typically needed for aerobic processes. This is generally achieved by aerating the medium.

The main advantages of batch or discontinuous operation are:

- Lower capital cost for equipment and automated systems
- Greater flexibility by varying the reaction time and operating conditions, such as temperature, pH, concentrations, etc.
- Greater adaptability to variations in influent contaminant concentrations and waste stream volume.

The main disadvantages of batch operation are:

- Non-productive idle time during filling, heating/cooling, and decanting operations
- Higher operating expenses, since pumps have to be operated to fill and empty the reactor, and controls are needed for maintaining reactor liquid level, requires analytical testing of each batch.

In the continuous mode, there is a continuous flow of liquid and cells into and out of the reactor.

The main advantages of continuous operation are:

- Lower reactor volume for a given production rate
- Consistent product quality, i.e., extent of biodegradation can be controlled by maintaining the operating conditions

The main disadvantages of continuous operation are:

- Lower flexibility in handling changing influent characteristics, since it is difficult to change operating conditions
- More process control to maintain operating conditions
- Higher capital costs

In the plug flow or tubular flow bioreactors the reactor contents are not well-mixed but rather flow in plug-flow fashion through the reactor. A concentration profile develops between the inlet of the plug-flow reactor, where the concentration of the contaminant is high, and the outlet of the reactor where the contaminant concentration is low. Due to the presence of this concentration profile in the reactor, the rate of biodegradation varies from the inlet to the outlet, which is different from the completely mixed or continuous stirred-tank system where the reaction rate is constant.

Table 3.1 gives a comparison of total reactor volumes required for achieving a specified removal efficiency for a first order biodegradation reaction:

Table 3.1
Reactor Volumes Required to Achieve Required Removal Efficiency

Number of reactors in series	90% removal efficiency	98% removal efficiency
1 CSTR	9.00	49.00
2 CSTRs	4.32	12.14
8 CSTRs	2.64	5.04
1 PFR	2.30	3.91

Note: Volume of each reactor expressed in units of Q/k

CSTR - Continuous Stirred-Tank Reactor

PFR - Plug-Flow Reactor

It should be noted that as the number of continuous stirred-tank reactors connected in series increases, the total reactor volume approaches the volume of a single plug-flow reactor.

3.2.2. Fixed Film Reactors

Fixed film reactors can be classified into two main types:

- Stationary fixed film reactors
- Moving fixed film reactors

In fixed film reactors the microorganisms are immobilized on the surface of a solid surface. The immobilized microorganisms, called a biofilm, are contacted with the liquid phase containing the contaminants. The contaminants diffuse into the biofilm, wherein the contaminants undergo degradation.

In the stationary fixed film reactor the solid phase is fixed and the liquid flows through the reactor in contact with the solid surface. In some cases, the biofilm is immobilized on the inside surface of hollow fibers or outside surfaces of solid tubes, and the liquid flows through the reactor contacting the biofilm.

In a stationary fixed film reactor the liquid phase can be completely-mixed or flow through the reactor in plug-flow fashion. The level of mixing in the liquid phase depends on reactor design.

A moving fixed film reactor consists of solid particles with the biofilm attached to it and suspended in the liquid phase. The liquid phase can be mixed mechanically using a mixer or the particles can be fluidized in the liquid phase, wherein flow turbulence causes the particles to mix in the reactor. The solid particles are settled from the liquid phase after exiting from the reactor and recycled back to the reactor inlet.

In a stationary fixed film reactor the biofilm stays in the reactor until it decays and sloughs off the solid phase and exits with the liquid phase. In a moving fixed film reactor, the biofilm stays in the bioreactor for a fixed time, depending on reactor design.

Examples of fixed film reactors includes:

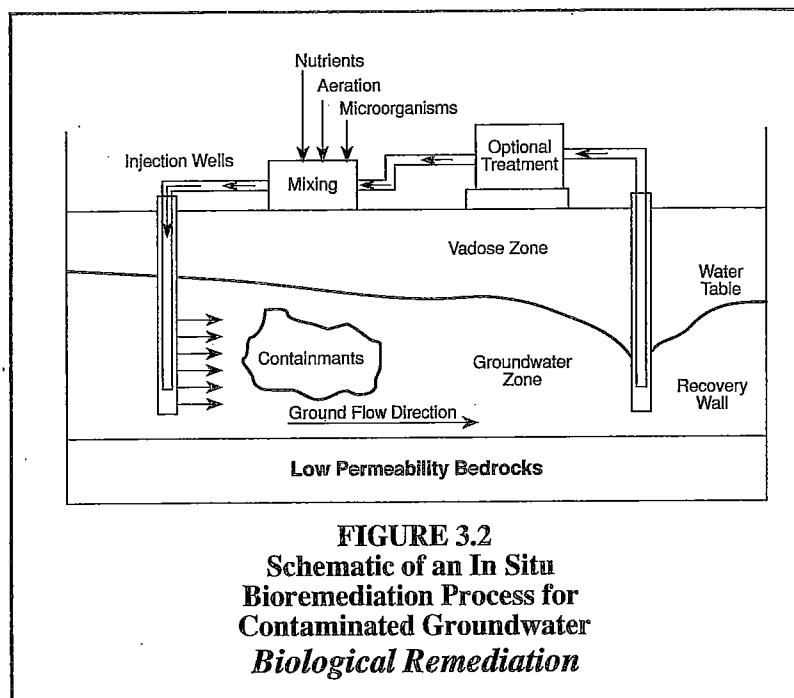
- Trickling filters
 - Biofilm immobilized on solid phase, and the liquid phase containing the contaminants trickles down through the packed bed
- Rotating biological contactors
 - Biofilm immobilized on rotating plates that alternately contact that water containing the contaminants and air as they rotate

- Submerged fixed film reactors
 - Biofilm immobilized on plastic packing and packed in a bed with the liquid phase containing the contaminants completely filling the void spaces bed may be considered fluidized.
 - Air may be pumped from the bottom of the bed
- Powdered activated carbon treatment systems
 - Biofilm immobilized on powdered activated carbon, which is mixed with liquid containing the contaminant

3.3. In-Situ Treatment

In-situ treatment can be applied to both inorganic and organic contaminants. In in-situ bioremediation, four main requirements must be satisfied as illustrated in figure 3.2:

- Presence of bacteria
- Availability of Terminal Electron Acceptor (TEA)
- Availability of nutrients
- Favorable environmental factors (e.g. pH, temp, moisture)



When indigenous bacteria are not present, suitable bacterial populations must be developed to effect biodegradation. TEA and nutrients have to be supplied for effective aerobic bioremediation. Distribution of the TEA, nutrients and microbial seeds is dependent on the soil property. A granular or lumpy soil results in the transport of these species to be diffusion limited resulting in a small growth rate. The problem is made worse in cases of soils with low permeability. Thus, in order to simulate microbial growth in subsurface environments, proper distribution of the TEA, nutrients and the microbial seeds must be ensured. Other environmental factors, such as

temperature, pH, and soil conditions, must be maintained in proper range to sustain biodegradation.

In-situ treatment can be applied to both saturated and unsaturated (vadose) zones. If the desired biodegradation is an aerobic process, oxygen must be supplied to the microorganisms. In the saturated zone oxygen can be supplied by saturating the water with oxygen and then injecting it into the ground. Although this technique has been demonstrated to some success for previous soils the method does not work effectively for soils with low permeability. Other means of supplying oxygen include addition of hydrogen peroxide, which decomposes to oxygen and water. If the process is anaerobic, other terminal electron acceptors such as nitrate or sulfate can be supplied to enhance biodegradation. An effective way to remediate soils in the vadose zone or in the top layers of the saturated zone, is to depress the water table to create a thicker unsaturated zone. It is easier and more efficient to remediate the residues on soil in the vadose zone as compared to the saturated zone, in view of the higher oxygen trapped in the soil pockets of the vadose zone as compared to the dissolved concentration in the saturated zone. In addition, contaminant mobility in the vadose zone is less stringent compared to the saturated zone. In the unsaturated zone, water pumping is not required as part of clean-up. Enhancement of biological activity in the unsaturated zone is achieved by passing water with dissolved oxygen or air through the zone.

Figure 3.2 shows the process for in-situ bioremediation of groundwater.

4. KINETICS OF BIOREMEDIATION

4.1. Bioremediation Phases

The various microbial growth and decay stages occurring during bioremediation can be visualized using a batch model, wherein the cell mass concentration as a function of time, may be represented by Monod kinetics. Figure 4.1 represents the biodegradation growth stages associated with biological remediation. Seven different growth phases can be distinguished:^{1, 2, 3}

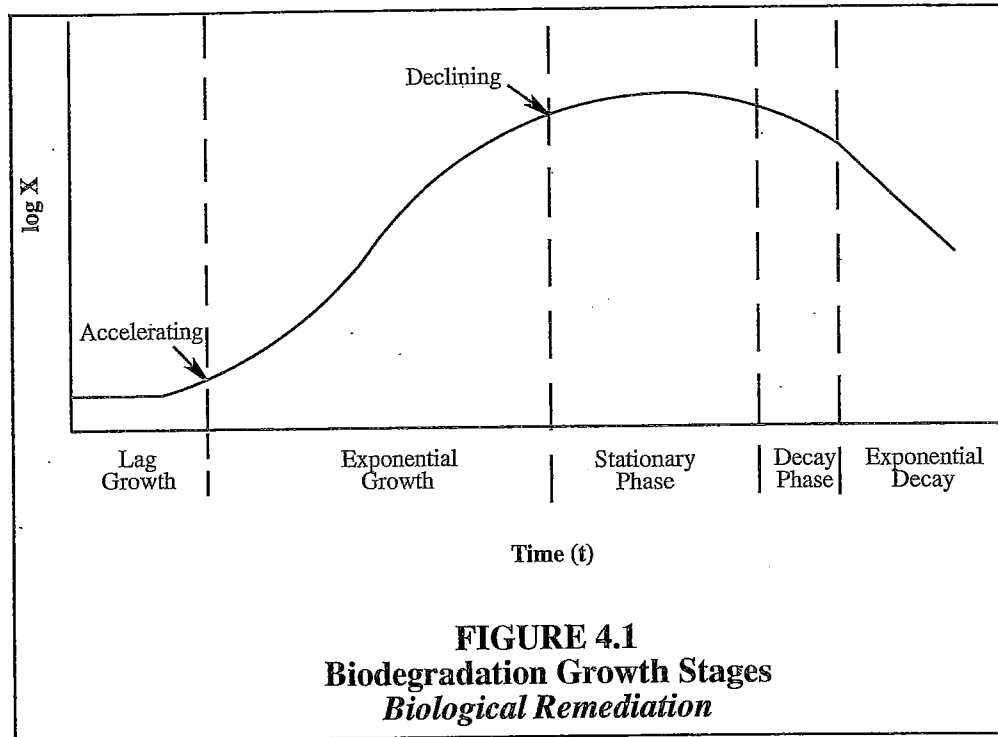
Starting (lag) phase - When microorganisms are initially exposed to a chemical, it takes them some time (time lag) to get acclimated to the environment in which the chemical is present

Acceleration phase - In this phase, when the cell has adjusted to the environment and starts to propagate, the microbes synthesize the growth factors and enzymes required for the degradation

Exponential phase - Both the cell concentration-time curve and cell-mass concentration-time curve depict an exponential function. This exponential relationship results from the basic fact that cell growth occurs by cell division, called binary fission, as mentioned earlier.

Hence, if the initial cell density is n_0 at time t_0 , then after m time divisions, the cell density becomes

$$n = n_0 * 2^m \quad (4)$$



Equation (4) can be rewritten as:

$$m = \frac{[\log(n) - \log(n_0)]}{(\log 2)} \quad (5)$$

The rate of increase in cell-mass concentration (x) can be written as follows:

$$R_x = \frac{dX}{dt} = \mu X \quad (6)$$

where:

R_X = the growth rate and μ is the specific growth rate

Equation (6) can be rewritten as:

$$\mu = \left(\frac{dX}{dt} \right) \left(\frac{1}{X} \right) \quad (7)$$

The specific growth rate, μ , is maximum when (dX/dt) is maximum, and can be expressed as:

$$\mu_m = \left(\frac{dX}{dt} \right)_{\max} \left(\frac{1}{X} \right) \quad (8)$$

Note that μ_m is constant during the exponential phase.

$$n = n_0 * 2^m$$

$$\log n = \log n_0 + m \log 2$$

Where; $m = t / k$

t = total time elapsed since the beginning of division

k = time required for one cell division

$$\log n = \log n_0 + \frac{t}{k} \log 2$$

Differentiating the above expression with respect to t gives:

$$\frac{1}{n} \frac{dn}{dt} = \frac{\log 2}{k}$$

n = the number of cells in a unit volume

Replacing n by x (the microbial density)

$$\frac{1}{x} \frac{dx}{dt} = \frac{\log 2}{k}$$

Defining the Specific Growth Rate μ as:

$$\mu = \frac{\log 2}{k}$$

$$\frac{dx}{dt} = \mu x$$

Equation (8) can be integrated using the boundary condition that $X = X_0$ when $t = 0$

$$X = X_0 \exp(\mu_m t) \quad (9)$$

The cell-mass doubling time, t_d , can be determined from equations (9):

$$t_d = \frac{\ln(2)}{\mu_m} \quad (10)$$

Declining phase - In this phase, the growth starts retarding, which is mainly due to the fact that the chemical used for growth has been depleted or the metabolite products are inhibiting further growth.

Stationary phase - Cell propagation is balanced by cell decay. The cell concentration reaches a plateau value.

Decay phase - The cell decay is higher than the propagation rate.

Exponential death phase - The number of living cells decreases exponentially, when cell growth or propagation has ceased completely.

4.2. Rate Constants and their Applicability

Standard methods for determining the biodegradability of a compound in the aquatic environment produce results in terms of percentage removal after a fixed time period.⁴ Although this can be useful for determining the "biodegradability" of a compound in the environment, it cannot be used for relative quantifying the rate of biodegradation. A quantitative expression for the rate of biodegradation is needed to calculate and predict the fate of the chemical in the environment and determine its distribution and concentration in the environment in the presence of other competing abiotic processes, such as adsorption and volatilization.

Quantitative measurements of intrinsic biodegradation kinetics, independent of the test system, are useful for developing reliable quantitative structure-biodegradability relationships, which enable biodegradation kinetics to be predicted for new chemicals and allow synthetic chemicals to be modified for enhanced biodegradability.^{5, 6}

The kinetics of biodegradation have been described by a variety of mathematical expressions, ranging in complexity and applicability. A review of some of the commonly used expressions is given below.³

First-order reactions:

If biodegradation is a first-order reaction, then the rate of substrate depletion (R) is proportional to the substrate concentration:

$$R = - \frac{d[S]}{dt} = k_1 [S] \quad (11)$$

where:

$-d[S]/dt$ = the rate of disappearance of the biodegrading chemical

t = time

k_1 = the first-order rate constant

$[S]$ = the concentration of the test chemical in the test system at any given time t

The rate of product (carbon dioxide) formation, can be mathematically described as follows:

$$R(\text{CO}_2) = \begin{cases} 0 & \text{for } t < t_d \\ a(1 - e^{-k_1(t - t_d)}) & \text{for } t > t_d \end{cases} \quad (12)$$

where:

- $R(\text{CO}_2)$ = the rate of carbon dioxide generation due to biodegradation
 a = the extent of biodegradation
 t_d = the lag time prior to onset of biodegradation due to acclimation.

Care should be exercised when using the half-life equation for describing biodegradation, since at low concentrations of the chemical, the rate of chemical transport can become limiting, and the reaction expression may not be sufficient to predict the temporal profiles of concentration. In such cases, substrate depletion, for example, may be governed by diffusive characteristics of the environment rather than the rate of reaction.

The rate of biodegradation, in the environment, also depends on the concentration of the microorganisms, responsible for biodegradation. However, if the concentration of microorganisms can be assumed to be constant in the test system, i.e., if the test chemical concentration is so small that growth of microorganisms can be ignored, then the rate constant, k_1 , in equation (12) incorporates the constant biomass concentration value. Variations in the quantity of biomass can change the rate constant, k_1 , and this is usually the reason for large variations in the reported values of the rate constant, k_1 , between different test sites. The use of first-order kinetics can be verified by checking whether k_1 varies (> 10 fold) over a wide range of test chemical concentration.

Representative values of the first-order rate constant, k_1 , are given in table 4.1.

Table 4.1
Representative First - Rate Order Constants

Chemical	k_1 (day ⁻¹)	Sample	Reference
Benzene	0.12	River	Lee and Ryan (1979)
Chlorobenzene	0.07-0.3	River	Bartholomew and
	0.01	Marine	Pfaender (1983)
4-Chlorophenol	0.035	River	Lee and Ryan (1979)
Methyl parathion	0.30	River Sediment	Cripe et al. (1987)

Second-order reactions:

When the concentration of the microorganisms does not remain constant in the test system, due to growth, then the rate of biodegradation may be written as follows:

$$R = -\frac{d[S]}{dt} = k_2[S][X] \quad (14)$$

where:

- | | |
|-------|--|
| k_2 | = the second-order rate constant |
| $[S]$ | = the concentration of the test chemical in the system |
| $[X]$ | = the concentration of the microorganisms in the test system |

The above second-order equation implies that the rate of biodegradation increases with the concentration of the test chemical and the biomass present. However, the rate of biodegradation reaches a plateau with increases in the test chemical concentration. A possible explanation of this phenomenon is, that as the substrate concentration is increased, it reaches a point where the environment becomes toxic for micro - organisms to reproduce. Hence, the above second-order equation is valid only in a narrow range of test chemical concentration.

It should be noted that the second-order equations involve the concentration of the microorganisms, $[X]$. This microbial concentration can be obtained from either plate count tests or other enumeration methods. It is generally assumed that:

- Plate counts (or other enumeration methods) detect similar percentages of the total microbial population in different samples;
- All, or a constant proportion, of the enumerated bacteria are transforming the chemical; and
- Transformation rates per cell are approximately the same for all sites examined.

The second assumption is usually valid for initial hydrolysis of the chemical, but generally does not hold for the "ultimate" biotransformation to carbon dioxide. For example, in the hydrolysis of pesticides, such as malathion, the enzymes involved in the hydrolytic biotransformation are present in most bacteria, and, hence, the concentration of bacteria as measured by enumeration is proportional to the hydrolytic enzyme concentration. The variation in the second-order kinetic constant, k_2 , as measured experimentally at different sites for the hydrolysis of malathion is small. Similar small site variations in k_2 values have been reported for primary biodegradation of phenol, para-substituted phenols, and various esters of 2,4-D in North American fresh waters. Generally, all the bacteria present in natural populations are not equally active in the degradation of a specific test chemical. Therefore, the use of a second-order equation, wherein $[X]$ is used as the concentration of the entire microorganism population is not always valid, and may lead to large site variations for the kinetic constant k_2 .

Monod Growth and Second-Order Kinetics

If the concentration of the microorganisms increases continuously due to growth resulting from the biodegradation of the test chemical, then the rate of biodegradation can be described by the following equation, first proposed by Monod (1949):

$$R = -\frac{d[S]}{dt} = -\left(\frac{1}{Y}\right) \left[\frac{[S][X]}{K_s + [S]} \right] \quad (15)$$

$$\frac{d[X]}{dt} = (\mu_{\max}) \left[\frac{[S][X]}{K_s + [S]} \right] \quad (16)$$

where:

- [X] = the concentration of the microorganisms
- μ_{\max} = the maximum specific growth rate
- Y = the yield coefficient (usually about 0.5 for aerobic cultures)
- [S] = the concentration of the test chemical
- K_s = a constant numerically equal to [S] at 0.5 μ_{\max} (i.e., the test chemical concentration at which growth occurs at half the maximum rate)

The Monod equations, as applied to enzyme-catalyzed reactions, were first proposed by Michaelis and Menten in 1913.

The specific growth rate of the microorganisms, μ , can be written as follows:

$$\mu = \left(\frac{1}{[X]} \right) \frac{d[X]}{dt} \quad (17)$$

The Monod equations have been successfully used to describe the rate of removal of synthetic chemicals in laboratory studies where the test chemical was the sole source of carbon and energy. However, it is important to note the limitations of the Monod equations.

The limitations of the Monod equations are as follows:

- It is assumed that the microbial growth occurs on the test chemical only and that the test chemical is the single growth-limiting chemical in the system. Thus, in in-situ applications where other chemicals present in the environment may compete for the growth substrate, Monod equation may not represent the microbial growth that accurately.
- The value of the yield coefficient, Y, is a constant. However, there are data which indicate that at low concentrations of the test chemical, a large amount of the energy derived from the biodegradation of the test chemical is used for maintenance energy requirements and not for growth
- Laboratory studies have also shown that many synthetic chemicals can be transformed by microorganisms which cannot use them as the sole source of carbon and energy. This phenomenon, called co-metabolism, is defined as the transformation of a non-growth chemical in the obligate presence of a growth chemical. The non-growth chemical is a compound that is unable to support cell replication

The Monod equation cannot be applied for the biological transformation of a non-growth chemical, since μ_{\max} and Y are zero.

4.3. Applicability of Various Kinetic Models

It has been proposed that the variety of substrate disappearance curves encountered in biodegradability testing is the result of interactions between substrate concentration, $[S]$, and population density, $[X]$, (Simkins and Alexander, 1984). To accommodate changes in population density, a mass balance equation is used to relate the population density and the substrate (test chemical) concentration.

$$[S_0] + \frac{[X_0]}{Y} = [S] + \frac{[X]}{Y} \quad (18)$$

where:

- $[S_0]$ = the initial concentration of the substrate (test chemical)
- $[X_0]$ = the initial concentration of bacteria
- $[S]$ = the test chemical concentration at a given time
- $[X]$ = the bacteria concentration

Combining the above equation with the Monod equations, yields a general differential equation, as follows:

$$R = -\frac{d[S]}{dt} = \mu_{\max}[S] \left(\frac{(S_0 - S)Y + X_0}{\{K_s + [S]\}} \right) \quad (19)$$

This equation can be simplified into simpler forms, as shown in Table 4.2. The conditions under which these models apply is also included in this table.

Table 4.2
Kinetic - Models for Biodegradation

Model	$R = -d[S]/dt =$	Conditions
First-order	$k_1 [S]$	$[S_0] < K_s$ $[X] = [X_0] = \text{constant}$
Second-order	$k_2 [S][X]$	$[S_0] < K_s$ $[X] = [S_0] + [X_0] - [S]$
Monod (no growth)	$k_0 [S] / \{K_s + [S]\}$	$[X]$ is constant $[S_0] > K_s$
Monod (growth)	equation (19)	$[X] = [S_0] + [X_0] - [S]$ $[S_0] > K_s$
Zero-order	k_0	$[S] \gg K_s$ bacteria saturated with test chemical
Logarithmic	$u_{\max} [X]$	$[X]$ is constant $[S] \gg K_s$ $[X]$ is not constant

At substrate concentrations below K_s (as would be the case for most synthetic chemicals in the aquatic environment), degradation can be described by either first-order equation, if the bacterial concentration is constant, or by a second-order equation, when the bacterial concentration is varying linearly, with time due to growth. The Monod (no growth) equations can be used when the initial concentration of the test chemical is higher than K_s , but if the bacterial yield coefficient, Y , is very low, the increase in bacterial concentration is small, i.e., the bacterial concentration can be assumed to be constant. The Monod (no growth) equation is the same as the Michaelis-Menten kinetic equation. The Monod (growth) equations are used when the initial test chemical concentration is higher than K_s and the bacterial concentration is varying due to growth. Zero-order kinetics can be used when the initial test chemical concentration is at least 50 times higher than K_s , and bacterial growth is small. Logarithmic kinetics is used when the initial test chemical concentration is significantly higher than K_s and bacterial growth occurs during biodegradation. Table 4.3 provides overall guidelines for the applicability of the various kinetic models, for various situations.

Table 4.3
Guidelines for Kinetic Model Applications

Chemical Concentration (M)	Situation	Model Type
$< 10^{-8}$	Environmental concentration	First-order
$10^{-6} - 10^{-5}$	Optimum analytical concentration	First-order Second-order Monod
$> 10^{-2}$	Spill concentration	Zero-order

4.4. Determination of Biodegradation Rate Constants

There are three types of experimental reactors that can be used to measure the kinetics of biodegradation. The three types of reactors are as follows:

- Batch reactor
- Continuous stirred tank reactor
- Fed-batch reactor

Batch Reactor System

In a batch reactor system nutrients needed for bacterial growth and water are well mixed in a closed reactor system. The concentration of the test chemical is monitored by analyzing the liquid contents periodically.⁵ If it is difficult to measure the concentration of the test chemical in the liquid phase, the test chemical is obtained as a uniformly ^{14}C labeled compound. The concentration of the chemical is monitored by measuring the radioactivity due to the ^{14}C using a scintillation counter. The rate of biodegradation can be calculated using the equation:

$$R = \left(\frac{[S_o]}{t} \right) \left(\frac{\text{dpm in cells} + \text{dpm CO}_2^{14}}{\text{dpm originally added}} \right)$$

where:

- R = the rate of biodegradation
- [S_o] = the initial concentration of the test chemical
- t = the biodegradation time
- dpm = the activity measured in disintegrations per minute (dpm) as measured by scintillation counting

The major problems in measuring biodegradation kinetics using a batch reactor are:

- Requirement of measuring liquid concentration of the test chemical periodically
- Difficulty in providing oxygen for aerobic cultures and removing the carbon dioxide that is produced

To circumvent this problem, attempts have been made to measure the oxygen uptake and to derive the biodegradation kinetics from the oxygen uptake measurements without measuring the liquid concentration. This has been achieved by using:

- Manometric respirometry
- Electrolytic respirometry

In manometric respirometry, the carbon dioxide produced by the biodegradation process is absorbed in soda lime pellets that are kept in a container located in the headspace of the batch reactor. As oxygen is used from the headspace, and the carbon dioxide produced is absorbed in the soda lime pellets, the total pressure in the batch reactor decreases, which is monitored manometrically. A large flask is usually used so that the oxygen available in the head space is sufficient for the entire biodegradation of the test chemical. The bio-kinetics using the kinetic equations is then derived from the oxygen consumption measurement.

In electrolytic respirometry, the batch reactor flask is connected to another flask, where a copper sulfate solution in water is electrolytically decomposed to produce oxygen. A pressure differential cell monitors the pressure in the batch reactor. When a finite pressure decrease occurs in the batch reactor, the electrolytic flask is triggered to produce oxygen, which then flows into the reactor flask. The pressure decrease in the reactor flask occurs due to the absorption of the carbon dioxide produced due to biodegradation, in the soda lime pellets that are kept in the head space of the reactor flask. The voltage needed to produce the oxygen in the electrolytic flask is monitored continuously, and this information is used to calculate the oxygen uptake in the reactor flask. The main advantages of using a batch reactor for measuring biodegradation kinetics are:

- Ability to handle volatile organic compounds, since the reactor system is closed
- Can be automated, as in the case of manometric or electrolytic respirometry

- Easy to conduct the experiment, since the reactor system is simple to operate
- No need for sampling and chemical analysis, as in the case of respirometry or when using a radio labeled compound

Measurement of oxygen consumption is one of the oldest means of assessing biodegradability. Time consuming manual measurement of oxygen uptake was gradually replaced by manometric respirometry and then later by electrolytic respirometry. A rather comprehensive review of the use of respirometers for the study of sewage and industrial waste biodegradation was published by.^{6,7}

Continuous Stirred Tank Reactor Systems

In a continuous stirred tank reactor system, a constant volume reactor is used with a continuous feed entering the reactor and a continuous waste stream leaving the reactor system. The reactor is usually well-mixed and samples are taken from the inlet and outlet liquid streams to monitor the rate of biodegradation. Oxygen needed for aerobic cultures is provided by saturating the influent stream with oxygen and the carbon dioxide produced in the reactor leaves in the dissolved state in the effluent stream.

Measurement of the test chemical concentration in the influent and effluent provides a direct measure of biodegradation. In some cases, the dissolved oxygen concentration can be measured at the inlet and outlet streams to measure the oxygen consumption in the reactor.

Continuous stirred tank reactor systems are more difficult to operate since a constant feed and effluent rate has to be maintained. This requires flow controllers, feed tank and effluent tank, level control, etc., making the entire system fairly difficult to operate. Furthermore, the test chemical can volatilize into the head space or adsorb on the surface of the microorganism cells, and these abiotic mechanisms have to be quantified before the rate of biodegradation can be measured.

Fed-Batch Reactor Systems

In a fed-batch reactor system, there is no constant influent and effluent flow, but rather the system is very similar to a batch system, except that a constant volume of feed stream is added to the reactor system at constant intervals of time, and an equivalent volume of the reactor contents is removed. This makes the fed-batch reactor system operate at a constant volume. Fed-batch reactor systems are easier to operate than continuous stirred tank reactors.

The rate of degradation determines, in part, the length of time needed to bioremediate a site. The extent to which a contaminant is degraded will determine if remediation goals are achievable. Bioremediation may not be a feasible option if the extent and/or the rate of degradation is low relative to the remediation objectives, and if it is not feasible to enhance the rate or extent by site modification. As discussed in the kinetics of bioremediation section, the rate of degradation is a function of contaminant concentration, microbial concentration, temperature, and other environmental factors.

5. SYSTEMATIC APPROACH FOR BIOREMEDIATION IMPLEMENTATION

5.1. Determining Contaminant Biodegradability

5.1.1. Screening for Biodegradability

In order to employ biodegradation as a remediation technology, the first step is to investigate the biodegradability of the compound of interest. A compound could be easily biodegradable resulting in complete mineralization, it could be persistent requiring specific conditions and presence of specific species and chemicals, or it could be recalcitrant indicating no degradation at all.

Simple experiments can be performed to test the biodegradability of the compound.

- A solution containing the test compound, as the sole carbon, energy source, and nutrients is inoculated with an acclimated microbiological culture.
- Temperature, pH, oxygen concentration and other important parameters are adjusted for the optimal yield and the culture is observed for microbial growth
- Results:
 - If the growth increases (usually turbidity is used as an indication of growth) substantially over a period of time then it may be inferred that the compound is biodegradable
 - If the turbidity does not increase for a considerable period of time a second assimilable carbon source is added
 - If growth is observed accompanied by reduction in the amount of test compound it implies that co-metabolism may be required for the successful biodegradation of the compound. *(A reduction in the quantity of a substance does not necessarily imply biodegradation. Other possible reasons for the decrease in quantity could be evaporation, sorption to cells, or apparatus etc. Thus all these possibilities should be examined before confirming biodegradation.)*
- A microbial culture may take months for acclimation and therefore sufficient time must be provided for observation before any conclusions are made.

The rates of mineralization (biodegradation) of some chemicals are directly proportional to their concentrations. Below a certain threshold concentration, usually mineralizable molecules are not degraded.² Microorganisms may not assimilate carbon from chemicals present in trace amounts in natural environments and will not grow or produce large populations needed for enhanced biodegradation. The minimum concentration of a chemical necessary to support microbial growth must be ascertained.

5.1.2. Estimating Biodegradation Potential for Contaminant

The chemical structure of the contaminant has a profound effect on its biodegradability and biodegradation kinetics. The environmental degradation of organic compounds involves a complex interplay of organism type, degradation environment, chemical structure, and biochemical reactions. Hence, isolating the affect of chemical structure on degradation is usually difficult, except when strict experimental protocols are used to minimize the impact of the other parameters.

Qualitatively, compounds containing highly branched alkyl chains degrade slowly when compared with straight chains. Functional groups, such as hydroxyl and carboxylate groups on benzene rings, increase biodegradability, while halogen, nitro, and sulfonate groups decrease biodegradability. Ortho-substituted aromatic compounds are slightly more biodegradable than para-substituted compounds, and the meta-substituted compounds have the least biodegradable substitution pattern. The information also suggests that compounds containing either pattern are resistant to biodegradation. Other qualitative relationships imply that, as a general rule, water soluble chemicals are usually more biodegradable than insoluble chemicals. These qualitative relationships have facilitated the synthesis and development of compounds which may be discharged to the environment with some degree of safety. For example, the replacement of alkyl benzene sulfonate surfactants with linear alkyl sulfonate surfactants in detergents has been based on biodegradability considerations.

Quantitative relationships between the structure of the chemical and its biodegradation characteristics appear rarely in the literature. In recent years, molecular descriptors, such as molecular surface area and volume, have been used in the prediction of biological properties. This is a result of the bioconcentration phenomena being partially related to the area of interface between the hydrophobic molecules and water. Early studies on quantitative relationships involved the correlation of biological activity with physiochemical properties, such as octanol-water partition coefficient, aqueous solubility, density, boiling point, and molecular weight. The processes by which the chemical becomes distributed and available in water are dependent on measures of its solubility. Hence, when solubility becomes rate limiting, correlations based on solubility become good indicators of biodegradability.

Recently attempts have been made to correlate quantum chemical descriptors with biodegradation kinetics. The ready availability of computing techniques has enabled the easy calculation of some quantum chemical parameters, such as atomic charge, electronic energy, ionization potential, and dipole moment ⁶.

Chemical structure of the contaminant is important in assessing its biodegradability. In a study conducted to develop an expert system for biodegradation assessment, the order in which the various groups contribute to biodegradability was determined, as is shown in the following list ⁸. The groups appear in a decreasing order of biodegradability:

Esters	Amide
Anhydride	Hydroxyl
Carbonyl	Epoxide
Site of unsaturation	Benzene ring
Methyl	Methylene

Hydrolyzable groups, azo bonds, halogens, and nitro groups are sites of anaerobic attack. Among the negative influences on aerobic biodegradability were molecular mass, branching, halogenation, and nitrogen heterocycles.

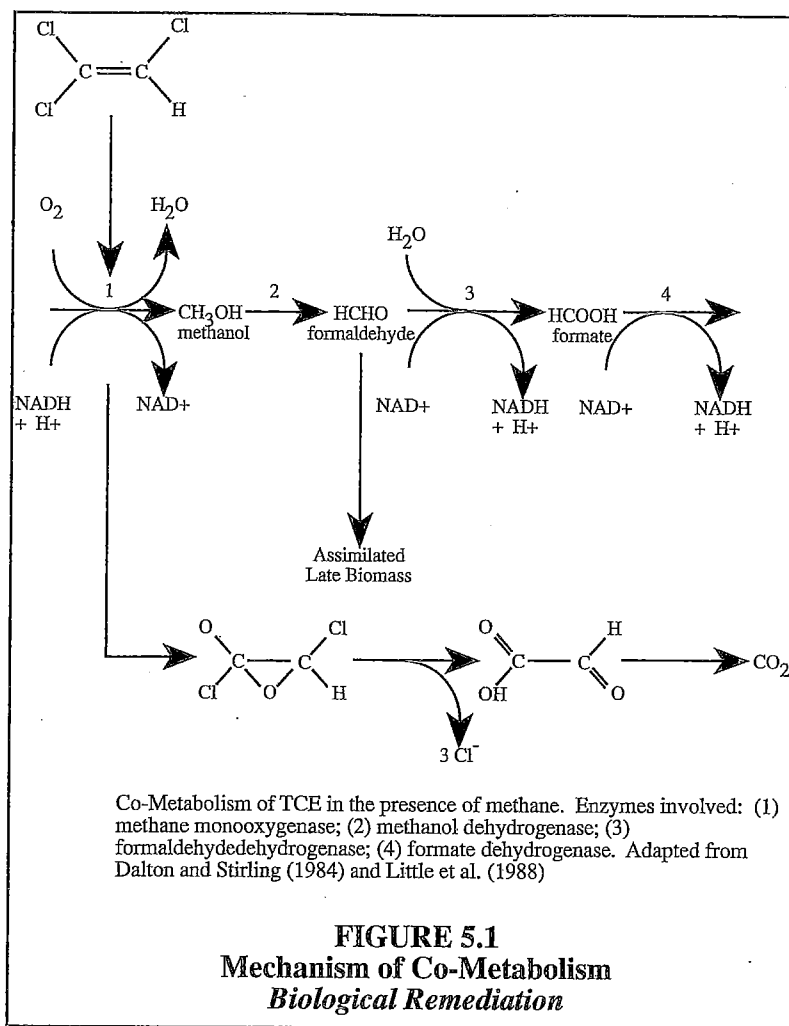
Catabolite repression, or substrate inhibition, occurs when a cell is faced with more than one utilizable substrate. Enzymes could be formed to catabolize all substrates, but this would be uneconomical for bacteria. Instead, enzymes are made to utilize the best substrate and only after exhaustion of the primary substrate other enzymes are formed which catabolize the "poorer" carbon sources. The most common type of catabolite repression involves the inhibition of formation of certain enzymes (i.e., repression) by the catabolite products of a readily utilizable carbon source. Catabolite repression has been historically known as the "glucose effect", because glucose is an easily assimilable substrate, and catabolite repression of other substrates was found to occur in the

presence of glucose. However, other easily utilizable substrates, such as acetate and citrate can also repress the utilization of other substrates.

The potential for both induction of enzymes and catabolite repression in a cell insures that inducible enzymes are produced only in the presence of substrate. If several substrates are present, only the enzymes acting on the most favorable substrate will be produced.

5.1.3. Possibility of Co-Metabolism

Co-Metabolism is the biotransformation of a compound by a microorganism that gains little or no energy for growth from the compound. It is a fortuitous transformation brought about by non-specific enzymes produced by the organism for other purposes. Examples are oxidation by co-metabolism of chlorinated aliphatic compounds, such as trichloroethylene, 1,2-dichloroethane, and vinyl chloride by methane oxidizers that possess methane mono oxygenases. A typical mechanism of co-metabolism is given in Figure 5.1 Other co-metabolizing microorganisms that possess broad substrate specific enzymes are toluene oxidizers, nitrifying bacteria, and ethane, propane, and ethylene oxidizers. The oxygenases produced by these microorganisms can initiate oxidations of a broad range of aromatic and aliphatic compounds under aerobic conditions. Degradation of TCE can also be brought about anaerobically. However, a degradation by product is vinyl chloride, which is more toxic than TCE itself. A typical mechanism for the anaerobic degradation of TCE is presented in Figure 5.2⁹. The addition of a carbon source is imperative when the degradative process is co-metabolic. However, more than one type of microorganism and/or carbon source can be used to co-metabolize a certain chemical reaction (e.g. trichloroethylene is co-metabolized when either methane or toluene is supplied as a primary carbon source.) In a field demonstration at Moffett Naval Air Station, methane addition was required for biodegradation of dissolved chlorinated ethenes introduced into an injection well. The concentration of trichloroethylene was diminished by 20 - 30% and vinyl chloride was reduced by 90 - 95% at a two meter distance downstream. When methane addition was ceased, 95% of the injected chlorinated ethene was detected in observation wells.



5.1.4. Enhanced Biodegradation

5.1.4.1. Nutrient Addition

In general, it is essential to maintain a minimum level of any required nutrient in order to support a desired microbe growth rate and sustain biodegradation. However, there is also a maximum nutrient level, above which substrate inhibition occurs. Hence, it is important to define these restrictive nutrient levels. Quantification of nutrients mineral needs is especially difficult because of the occurrence of metal complexes, some cellular and other non-cellular in origin. Excessive amounts of essential nutrients such as PO_4^{3-} and SO_4^{2-} also can exert catabolite repression that affects specific metabolic pathways while not affecting growth.

A first approximation of the minimum requirements in nutrient design is based on the stoichiometry of the overall biodegradation process:



Various empirical formulas have been proposed to represent the organic composition of microbial cells. One of the oldest and most widely accepted in the field of wastewater treatment is $C_5H_7O_2N$. Another formula which includes phosphorus is $C_{60}H_{87}O_{23}N_{12}P$. When microbial cells are incinerated, the ash left after incineration, includes potassium, calcium, magnesium, sodium, iron, and trace amounts of manganese, cobalt, copper, molybdenum, and zinc. Although these elements are known to play a role in metabolism, the specific contribution of each element is unknown, and elemental constants varies widely.

Generally nutrients can be classified into Macronutrient minerals, which include potassium, magnesium, calcium, iron and sodium, and Micro-nutrients minerals, which includes cobalt, zinc, molybdenum, copper and manganese.

Using the above empirical formulas for biomass, the amounts of Nitrogen source and Phosphorus source can be calculated using the overall stoichiometry equation. Generally the amount of phosphorus required is about one-fifth of the nitrogen requirement.

Nitrogen can be introduced in the nutrient media in either inorganic or organic forms. The most common inorganic forms are as ammonium or nitrate ions. Ammonium ions can be assimilated directly by the cell through amination of a keto acid to form glutamic acid and other amino acids, which can be incorporated into proteins and nucleic acids. When nitrate is supplied as the only nitrogen source, it must be first reduced to ammonia before the bacterium can use it.

Phosphorus is an essential component of nutrient media, since insufficient phosphorus will limit growth and prevent the complete removal of organic substrate in a manner similar to insufficient nitrogen. Usually the phosphorus is added in the form of inorganic orthophosphate.

Biodegradation can be limited by the availability of nutrients and hence the application of nutrients (sufficient nitrogen, phosphorus and mineral media) can enhance the rate and extent of biodegradation.

Mineral nutrients, in particular, nitrogen and phosphorus, are often limiting in the subsurface environment and must be supplied to ensure biodegradation of a large contaminant mass. For example, Mulkins-Phillips and Stewart found that phosphorus limited the rate and extent of growth of a *Norcadia* sp. on 1% (volume/volume) Bunker C fuel oil. Prior to beginning an *in situ* bioremediation project in Ambler, PA, laboratory experiments indicated that the native microorganisms could be stimulated by the addition of inorganic nitrogen, phosphate salts, and air. The carbon:nitrogen:phosphorus ratio necessary to enhance bioremediation can vary between 100:10:1 to 100:1:0.5, depending on the methodology used and the phase of the contaminant (liquid or solid phase).

Addition of nutrients is not always necessary. For example, in bioventing applications, nutrient addition is not necessary when the soil matrix contains adequate amounts of nitrogen and phosphorus. Nutrients may be needed to sustain increased rate of degradation as bacteria populations increase.

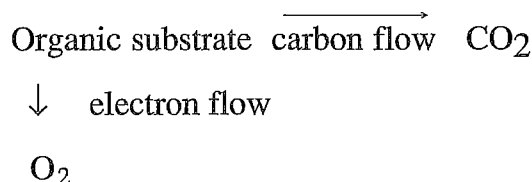
5.1.4.2. Application and Feasibility of Alternate Terminal Electron Acceptors

An organic substrate is not directly oxidized to carbon dioxide and water in a single chemical step because there is no energy conserving mechanism that could trap so large an amount of energy. Consequently, biological oxidation occurs in small steps. Basically, oxidation requires the transfer of an electron from the substance being oxidized to some acceptor molecule which will subsequently be reduced. In most biological systems, each step in the oxidation of the substrate

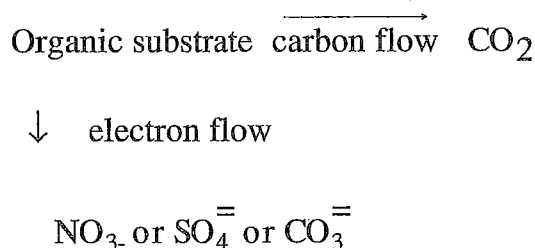
involves the removal of two electrons and the simultaneous loss of two protons. The electrons and protons are transferred to an acceptor molecule. The acceptor molecule will not accept the protons until it has accepted the electrons, and thus it is referred to as an electron acceptor.

In the biodegradation of organic substrates, the terminal electron acceptor is an inorganic molecule, such as oxygen, nitrate, sulfate or carbonate. Depending on the type of electron acceptor, the degradation can be classified as either aerobic or anaerobic.

Aerobic Respiration:



Anaerobic Respiration:



Depending on the conditions under which the biodegradation has to be conducted, alternate electron acceptors, such as nitrate, sulfate or carbonate, can be added to replace oxygen. In subsurface environments, where oxygen availability is limited, biodegradation using alternate electron acceptors can be conducted (for example, microbial degradation of acenaphthene and naphthalene has been demonstrated using NO_3^- as the TEA in soil-water systems¹⁰).

Aerobic biodegradation is always preferred because it is faster than anaerobic processes. (Bioremediation is cost-effective only if it occurs rapidly, due to increased operation and maintenance cost over extended time. Regulatory agencies will also require rapid remediation to remove the source of continuing contamination.) For example, the biodegradation rate constant for carbofuran in soil is 0.047/day aerobically and 0.026/day anaerobically. Methods for increasing oxygen availability include air sparging, addition of pure oxygen, and addition of hydrogen peroxide during *in situ* bioremediation. The low solubility of oxygen in water limits the amount of oxygen that can be provided during *in situ* bioremediation.

The use of alternate electron acceptors has also been used in bioremediation. Nitrate addition is a method of supplying both a mineral nutrient and an electron acceptor.

5.1.4.3. Effect of pH

The pH, a measure of the hydrogen ion (H^+) activity, is particularly important as a parameter of microbial growth. The rate of biodegradation is influenced by the pH, the optimum pH and range of pH over which growth can occur being determined by the nature of the organisms present in the system. It is possible to make some generalizations that are useful in defining a chemical environment for the cell. Most bacteria have a pH optimum (i.e., that pH at which growth rate is

maximum) around 6.5 - 7.5. Most environments have been found to have a pH in the physiological range (i.e. between 5 - 8.5). Most microorganisms do not grow effectively in acute acidic ($\text{pH} < 5$) or basic ($\text{pH} > 8.5$) conditions.

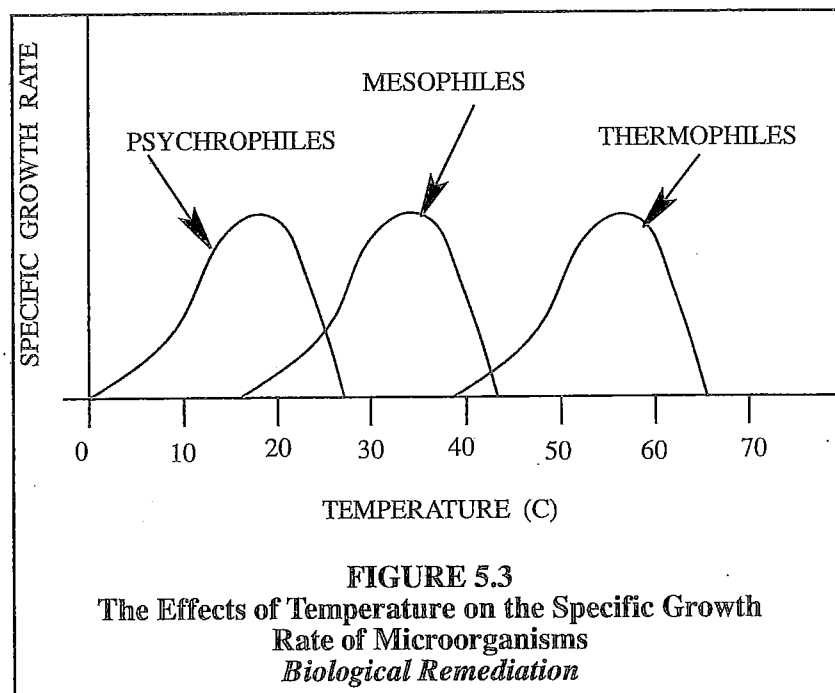
Changes in pH occur due to microbial activities: a hydrogen ion is generated during uptake of ammonium ion (NH_4^+), and a hydrogen ion is consumed during NO_3^- metabolism, etc.

While control of pH is important to the maintenance of an optimal environment for microbial degradation, monitoring of pH can yield useful information about the rate and extent of degradation.

5.1.4.4. Effect of Temperature

Microbial growth rate, as with all chemical reactions, is a function of temperature. In general, microorganisms will grow over a temperature range of 25 to 30° C. However, it is important to note that there are microorganisms in the environment, that will grow at temperatures below 0° C and above 93° C — the primary requirement is availability of liquid water.

Figure 5.3 shows the effect of temperature on three groups of microorganisms: the psychophilic, mesophilic, and therophilic.



There are three generalizations that can be made concerning the effect of temperature on growth rate, and hence the rate of biodegradation. First, growth occurs only over a 30° C range for a microorganism: second, growth rate increases slowly with increasing temperature until the maximum growth rate is reached; and third, above the temperature for the maximum, growth rate falls rapidly with further increase in temperature.

The effect of temperature on the specific growth rate (refer to equation (7) in section 5.1.8) can be written as follows:

$$\mu = A \exp (-E_a/RT)$$

where:

- A = the Arrhenius constant
- E_a = the activation energy (kcal/mole)
- R = the universal gas constant (1.987 cal-mol)
- T = the absolute temperature

Typical values for activation energy for microbial growth are 13-17 kcal/mole. Above the maximum temperature for growth, the overall growth rate falls very rapidly, and is characterized by activation energies of 70-90 kcal/mole. The value of Arrhenius constant A, varies for different microorganisms. For the case of E. coli for example, for growth rates between 18 and 46 °C the value of the Arrhenius constant has been reported to be 2.92×10^{11} . For higher values of activation energy in temperature ranges above the maximum possible for growth research indicates, that for the same increase in temperature, the rate of inactivation is faster than the growth rate.

In *in situ* bioremediation, it is critical to know the environmental temperature and its variability since the efficacy of using microbial degradation processes varies with temperature. Usually the temperature at a given site may vary from freezing temperatures in the winter to about 30° C in the summer season. Accurate prediction of microbial growth rate requires an analysis of the monthly variation in the temperature, and the temperature variation with depth below the surface. Usually in the Northeast, bioremediation is dormant during the winter and only a nine month period is effectively available. In view of these observations an average growth rate may not be representative of the true growth rate. A first cut estimate, however, can be obtained by evaluating the growth rate for the average year round temperature.

5.1.5. Determining the Limiting Criteria

For determining the rate of biodegradation, the rate limiting step has to be investigated. In a typical subsurface, factors like dissolved oxygen and the availability of substrate to the microorganisms may be limited. Depending on the conditions of the system, different processes like diffusion, adsorption-desorption, or dissolution-precipitation may control the reaction. Biodegradation modeling is required to predict the rate of attenuation under the specific conditions. The model should be developed for the conditions which can be generated in the subsurface environment. A verification of the model predictions should be made by generating the model conditions in the subsurface environments and then measuring the rate of biodegradation.

One such model is developed by Rifai and Bedient, which compares a oxygen transport limiting rate condition and dual substrate Monod kinetic relationship to calculate biodegradation. The first condition assumes an instantaneous reaction between organics and oxygen, thus, assuming that the reaction is oxygen transport limited. The second condition, which is more accurate but at the same time more complex, utilizes a dual substrate Monod kinetic relationship to predict the biodegradation rate. Results of this analysis indicate that the instantaneous reaction is an adequate assumption when non-dimensionalised reaction rates are large.

Substrate availability could be the rate limiting step in some cases. In order to convert the substrate to biomass, it must be transported to the microbial cell. If the substrate is soluble, then it can be transported through the aqueous phase. If, however, the substrate is not soluble, then physical contact with the microbial cell is essential. In this case, diffusion becomes the operative mechanism. In some cases, an extra cellular enzyme is secreted by the microbes which emulsifies

the substrate assisting diffusion through the cytoplasmic membrane. Thus, the form in which the substrate is available to the microbes is important. Additional forms could be as emulsion, vapor, or availability through an inert carrier.

5.1.6. Biodegradation Products

The products of biodegradation are not always carbon dioxide and water. TCE, for example, degrades to vinyl chloride which is more toxic than TCE itself. Biodegradation is a multi-step process, and the fate of the products formed in each step depends on their biodegradability and toxicity.

It is important to know the identity and amount of by-products that may be produced due to biodegradation. In certain cases the degradation products can pose a greater environmental hazard than the parent contaminant itself. If the by-products can not be treated either in sequence or concurrently with the bioremediation process, it may not be feasible to use bioremediation.

5.2. Developing the Microbes

5.2.1. Microcosm Stimulation

Adaptation of microorganisms to the environment is essential for ultimate bioremediation of the contaminants. This is usually achieved by applying essential nutrients and oxygen (for aerobic cultures) to the system in order to stimulate the natural microbiota. Both nutrients and oxygen are needed for acclimation and growth of the microorganism culture. When the culture is stimulated, the cells undergo growth through cell division.

5.2.2. Enrichment, Isolation, and Estimation of the Desired Species

Microorganisms having desirable characteristics, such as acclimation to certain contaminants, may be isolated from natural sources. Trained microbiologists are required to isolate, purify, screen, and test a culture from a natural source.

Culture enrichment involves the transfer of soil, sewage, or some other materials with a large and diverse population of microorganisms into a selective medium followed by incubation of the culture under such conditions of temperature, aeration, and pH, among others, that the growth of desired forms is favored. A small amount of the initial culture is transferred to a second that is set up in the same manner as the first, and this procedure is continued until the desired flora predominates and can be isolated in pure culture. This culture enrichment technique is based on free competition between the different organisms in liquid media. If a liquid selective medium is inoculated with a variety of organisms and incubated under any chosen set of conditions, these organisms for which the environment is most suitable will outgrow all others and eventually become predominant.

As listed in Table 5.1, for the enrichment of many organisms a basal medium of the following composition can be applied:

Table 5.1
Composition of Basal Medium

<i>Basal Medium</i>	
K ₂ HPO ₄	1 g
MgSO ₄ ·7H ₂ O	0.2 g
FeSO ₄ ·7H ₂ O	0.05 g
CaCl ₂	0.02 g
Trace element solution	0.1 mL
Water	1 liter

The requirements for the carbon, nitrogen and energy sources depend on the type of organism to be enriched. The trace element solution is usually applied to prevent any mineral deficiencies, and usually consists of very dilute quantities of trace salts, such as AlCl₃, KI, KBr, etc. Carbon dioxide is not only needed by those organisms that use it as the sole carbon source, but also by various chemoheterotrophs. It is not usually added to aerobic cultures, but should be included in growth media for anaerobes. It is generally added as NaHCO₃.

In addition, growth factors are added as supplements to the enrichment media for a large variety of microbes. These growth factors include vitamins, such as B₁₂, complex mixture of vitamins, and amino acids. Growth-factor requirements can often be met by including a small amount of yeast extract (0.001 to 0.05%) to the enrichment medium.

The enrichment culture is one of the most powerful techniques available to the microbiologist. Almost infinite number of permutations and combinations of the various environmental variables (nutrient composition of the medium, aeration, pH, temperature, etc.) can be used for the isolation of specific microorganisms from natural sources.

Isolation is the separation of a particular microorganism from the mixed populations that exist in nature. Isolation involves not only the separation of the pure culture from a mixed microbial population but also its maintenance in an artificial environment where it is protected from contamination by other microorganisms. The isolated culture vessel (usually a test tube, Petri dish, or flask) is initially sterilized (free of any living microorganism) and kept in a sterile room.

Isolation of the pure culture is generally achieved by:

- Plating methods
- Dilution methods
- Single cell separation

Plating methods: This method involves the separation and immobilization of individual organisms onto a solid nutrient medium solidified with agar or some other appropriate jelling agent. Each organism type grows to a visible colony from which organism can be readily isolated. The agar concentration used in plate culture is generally 1-2%.

The streaked plate generally is the most useful plating method. A sterilized bent wire is dipped into a diluted mixed suspension of organisms and is then used to make a series of parallel, non-overlapping streaks on the surface of the solidified agar plate. The inoculum (the microbial material used to seed or inoculate a culture vessel) is progressively diluted with each successive streak, so that the latter streaks can result in well-isolated colonies. The solid medium prevents migration of organisms and helps in forming isolated pure colonies.

Alternatively, isolations can be made with poured plates: successive dilutions of the inoculum are placed in sterile Petri dishes and mixed with the molten but cooled agar medium, which is then allowed to solidify. Colonies subsequently develop embedded in the agar.

Dilution Method in a liquid medium: Some of the larger-celled bacteria have not been successfully cultivated on solid media, and many protozoa and algae can only be grown in a liquid medium. The simplest procedure for isolation in a liquid medium is the dilution method. The inoculum is subjected to serial dilution in a sterile medium, and a large number of tubes of medium are inoculated with portions of each successive dilution. The purpose of this operation is to inoculate a series of tubes with a microbial suspension so diluted that the probability of introducing even one individual microorganism into a given tube is very small: a probability of the order of 0.05. The fraction of tubes that receives no microorganisms is therefore 0.95, the fraction receiving one microorganism is 0.048, the fraction receiving two organisms is 0.0012, the fraction receiving three organisms is 0.00002, etc. As a result, if a tube shows any subsequent growth, there is a very high probability that this growth has resulted from the introduction of a single organism. The dilution method has a major disadvantage that is that it can only be used to isolate the numerically predominant member of a mixed microbial population.

Single-cell isolation: When neither plating nor dilution methods can be applied, microscopically controlled isolation of a single cell or organism from a mixed population is used. The technical difficulty of single-cell isolation is inversely related to the size of the organism which one wishes to isolate. In the case of large microorganisms, purification involves the capture of a single individual in a fine capillary pipette and the subsequent transfer of this individual through several washings, in relatively large volumes of sterile medium, to eliminate microbial contaminants of smaller size.

For smaller microorganisms, a micromanipulator, which is a mechanical device, is used in conjunction with very fine glass operating instruments. Microbe cultures developed for injection for remediation applications are commercially available.

———5.3. Introduction of the Microbes to Site

Depending on the type of site, the microbes can be introduced into the site in many ways. In the case of soil bioremediation, the microbes can be added to the nutrient media which is then sprayed evenly on the site or injected into the subsurface vadose zone through a grid of injection ports. In these cases, the transport of the microbes is dependent on the nutrient flow in the site. The transmissivity of the soil plays a significant role in the nutrient distribution and transport of the microbes to the site contamination. Low soil transmissivity limits the feasibility of soil bioremediation. If the site is a liquid mass, such as a lake or lagoon, then the microbes can be added directly and distributed very easily by natural convection or pump-around systems.

———5.4. Optimum Conditions for Bioremediation

The essential requirements for bioremediation are:

- A viable inoculum
- An energy and carbon source
- Essential nutrients
- Absence of growth inhibitors
- Appropriate physico-chemical conditions

Normally, a viable inoculum exists in any natural environment. The indigenous microorganisms present in the natural environment may not be acclimated to the contaminants, but are usually

viable. Appropriate physico-chemical conditions, such as temperature, pH, etc. may have to be adjusted to allow biological growth to occur. Usually, it becomes essential to add the required nutrients, supply oxygen (for aerobic degradation) and some additional carbon source for initial stimulation of the microorganisms and allow acclimation to occur.

Defining the optimum conditions for effective biodegradation usually involves selecting the proper nutrients, maintaining necessary supply of oxygen for the aerobes, pH adjustments, and applying the needed carbon and energy sources. The basic objective is to maintain the growth of the microorganisms based on the degradation of the contaminants, at its maximum value. In Table 5.2 the necessary conditions for microbe growth are collected.

Table 5.2
Specific Requirements of Microbes for Soil Bioremediation ¹³

Environmental conditions necessary for bioremediation:	
pH	5.5 - 8.5
Moisture	25 - 85% of water holding capacity
Oxygen	Greater than or equal to 0.5 ppm
Temperature	10 - 45°C for most applications
Nutrients	Ratio of Carbon:Nitrogen:Phosphorus 120:10:1

5.5. Selection of Bioremediation Process

Bioremediation processes can be classified into four types. In the following sections, each bioremediation process, its feasibility, and its cost will be discussed in detail. Factors to be considered in determining whether or not bioremediation is feasible at a site include:

- Contaminant characteristics
- Microbial characteristics
- Site conditions and contaminated medium physical characteristics
- Regulations governing the implementation of bioremediation
- Public health and safety issues
- Time required to achieve remediation

5.5.1. Above Ground Treatment of Contaminated Soils (Landfarming/Composting)

5.5.1.1. Land Farming

5.5.1.1.1. Feasibility and Conceptual Approach

Organic contaminants in soils are susceptible to aerobic digestion, and this process occurs naturally to a limited degree. The rates may be greatly enhanced by fertilizing, irrigating, and tilling the soil to increase the availability of nutrients, moisture, and oxygen to the soil microorganisms. This process of fertilizing, irrigation and tilling the soil to enhance bioremediation is called land farming

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In land farming, the micro-organisms used are most often the indigenous population, unless the environment is too severe or biodegradation of specific contaminants requires specially-developed microbes. Through scheduled tilling and maintenance of the moisture content, pH, and nutrients, decomposition and immobilization of the contaminants occur within both the upper soil layers (zone of incorporation) and the underlying layers. Usually, landfarming is effective for 18-24" thick layer of contaminated soil. Treatment of layers >24" by landfarming is rare and has limited success.

Land farming has been used successfully throughout the United States, especially at petroleum refinery sites and also with creosote contaminated sludges and soils.

5.5.1.1.2. Factors Affecting Land Farming

Factors affecting land farming can be classified into three categories:

- Substrate limiting factors
- Environment limiting factors
- Biological consortia limiting factors

Substrate limiting factors are as follows:

- Substrate biodegradability
 - Organic substrate must be biodegradable, under aerobic conditions. Some compounds, such as PCBs are extremely bioresistant, and hence are not suitable for land farming techniques. For contaminated soils containing bioresistant compounds, pretreatment is required. Pretreatment schemes include supercritical extraction, solvent washing, and chemical dechlorination, as in the case of PCBs.
- Substrate toxicity
 - Some organic compounds are toxic to the microorganisms above a limiting concentration level. In some the cases, substrate toxicity can be controlled by dilution or pretreatment.

Environment limiting factors are as follows:

- Temperature
 - Temperature is an important environmental limiting factor. For successful land farming, temperatures favorable for microbial growth (15 - 45°C) are needed
- pH
 - pH needs to be maintained between 5 - 8 for effective bioremediation. pH control is needed where acidic (i.e. soils are present)
- Nutrients
 - For land farming operations, a C:N:P ratio of 100:2:0.2 has been found to be successful

Biological consortia limiting factors include:

- Diversity
 - The diversity of microorganisms present allow a wide variety of organics to be degraded successfully. The diversity of microorganisms present at the land farming site will control the rate of bioremediation and the kind of chemical contamination that can be treated

- Acclimation
 - Acclimation processes will delay the onset of biodegradation. However, natural evolutionary processes will result in acclimation of the indigenous microbes

5.5.1.1.3. Advantages/ Disadvantages

The major advantages of land farming are as follows:

- Simple to implement
 - The land is merely covered evenly with the contaminated soil, periodically dosed with nutrients, and mixed.
- Less expensive bioremediation technology.
- Applicable for slowly degrading contaminants, which would render bioreactor approaches infeasible due to large bioreactor sizes and costs.
- Usually employed for moderate to low contaminate concentrations.

The major disadvantages of land farming are as follows:

- Volatile contaminants, such as solvents, etc., must be pretreated, since they would evaporate into the atmosphere, causing air pollution.
- Particulate matter is also a concern because it may cause a dust generation problem.
- Presence of toxic metal ions requires pretreatment to avoid toxicity to the microbes and possible leaching from the contaminated soil into the ground.
- Land farming requires proper control of pH, water content, and nutrients.
- Can only be used where environment temperatures are favorable during most of the year.
- Can only be used for aerobic treatment of the organic waste.
- Requires a large area for treatment if significant volume of contaminated soils are present.
- May require bottom lining of large area if concern that leaching of contaminants may further degrade ground water.
- Less rapid method of remediation compared to other bioremediation techniques.

5.5.1.1.4. Site Characteristics For Selection of Land Farming

Land farming can be used when environmental conditions are suitable such as favorable temperatures for most of the year, reasonable rainfall, to provide moisture to the site, and availability of land.

5.5.1.1.5. Approximate Costs

Conventional land treatment costs range from \$50 to \$80/cubic yard. For 6,000 cubic yards of a soil contaminated with diesel fuel, land treatment in an unlined system, requiring no excavation, costs about \$33/cubic yard. These are the costs from a specific case study. In general for a variety of petroleum contaminated sites the costs are usually between \$30 - 50/cubic yard for sites containing greater than 1000 cubic yards of contaminated soils.

5.5.1.1.6. Additional Issues and Research Needs

Land farming is a well-established bioremediation technique that has been successfully used for a variety of applications. Experience and empirical relationships usually are relied upon for design. More research is needed toward the scientific development of landfarming techniques and broader applications. The major issues and research needs that need to be addressed are:

- Possible contamination of the environment from the degradation by-products
- Quantification of degradation kinetics
- Enhancement of degradation capabilities by addition of plasmids, to enable fast acclimation
- Monitoring of the land farming site, to obtain quantitative progress measurements

5.5.1.2. Composting

Composting is defined as the biological decomposition and stabilization of organic substrates under conditions which allow development of thermophilic temperatures as a result of biologically produced heat, with a final product sufficiently stable for storage and application to land without adverse environmental effects. Thus composting is a form of biological waste stabilization, but one that requires special conditions of moisture and aeration to produce thermophilic temperatures

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Composting is usually applied to solid or semisolid materials. (Approximately 800 million tons of solid and semisolid wastes are generated in the United States per year, and over 130 million are actually collected.) The process can be conducted under aerobic conditions, i.e., in the presence of air or oxygen, with carbon dioxide, water and heat being the main products of biological metabolism. Alternately, composting can be conducted under anaerobic conditions, with the metabolic products being methane, carbon dioxide, and numerous intermediates, such as low-molecular weight organic acids.

The objectives of composting have traditionally been to biologically convert putrescible organics to stabilized form and to destroy organisms pathogenic to humans. Composting can also effect considerable drying, which is of particular value with wet substrates, such as municipal and industrial sludges. Decomposition of substrate organics together with drying during composting can reduce the cost of subsequent handling and increase the attractiveness of compost for reuse or disposal.

Compost can be disposed of more easily than untreated solid waste. If the product is reusable, it can accomplish a number of additional purposes including. (However, when applying stabilized compost to soil one must be careful about plant uptake of undergraded residuals.):

- To serve as a source of organic matter for maintaining of building supplies of soil humus, necessary for proper soil structure and moisture holding capacity
- To improve growth or vigor of crops in commercial agriculture or home-related uses
- To reclaim and use valuable nutrients including nitrogen, phosphorous, and a wide variety of essential trace elements

Composting systems can be classified into two types: reactor systems and nonreactor systems.

Reactor composting systems:

Reactor systems can be further classified into vertical, horizontal and inclined flow reactors. Vertical flow reactors can be further defined based on the bed conditions in the reactor. Some systems allow for agitation of solids during their transit through the reactor, and are called agitated bed reactors. In other systems, the composting mixture occupies the entire bed volume and is not agitated during its residence time in the reactor. These are called moving packed bed reactors and can be fed on either a continuous, intermittent, or batch basis.

Horizontal and inclined flow reactors have been divided into those which employ a rotating drum (tumbling solids bed reactor) and those which use a bin structure and varying method of agitation, using either forced aeration or mechanical agitation.

Nonreactor composting systems:

Nonreactor composting systems are those that do not use a reactor, and can be further classified into agitated solids bed and static solids bed. An agitated solids bed disturbs or breaks up by some manner the composting mixture during the compost cycle. This may be achieved by periodic turning, tumbling, or other methods of agitation. The windrow system is an excellent example of a nonreactor agitated solids bed system. Mixed compost material is placed in rows and turned periodically, usually by mechanical equipment. The height, width, and shape of the windrows vary depending on the nature of the feed material and type of equipment used for turning. Oxygen is supplied by gas exchange during turning and by natural ventilation resulting from buoyancy of the hot gases in the windrow. In some systems, forced aeration is used for enhanced oxygen transfer.

The major limitation of composting for treating hazardous wastes is the lack of experimental data for a wide variety of toxic organics. Composting has been applied to wastes, such as TNT, petroleum sludges, and certain pesticides.

Major nuisance problems associated with composting are odors and dusts. Dust is also a problem for other landfarming techniques. Concern over odors has in recent years become of paramount importance in siting composting facilities and process selection.

5.5.1.2.1. Approximate Costs

Windrow composting, using recycled compost product, can usually be conducted for less than \$20-40/dry ton, including land purchase, site preparation, mobile equipment, concrete pad, fixed roof cover, controlled aeration system, and operation and maintenance expenses. Static pile composting with wood chips is considerably more expensive, primarily because of the cost of wood chips. Assuming chip costs of \$13/m³, the cost of the new chip replacement alone is about \$42/dry ton of sludge. Total system costs are usually in the range of \$60-120/dry ton, the major cost items being the bulking agents and labor.

5.5.2. Above Ground Slurry/Soil Treatment

5.5.2.1. Feasibility and Conceptual Approach

A typical soil-slurry treatment reactor system is shown in Figure 5.4 The process consists of the following steps:

1. Feed addition system: The contaminated soil mixed with water, nutrients, and other pH adjusting solutions, is pumped into the bioreactor
 2. Aeration system: An aeration blower is used to pump air through the slurry phase.
-

3. Emission control system: The air leaving the bioreactor has to be treated to remove volatile contaminants. Typical air emission treatment systems include carbon adsorption and/or biofilters
4. Bioreactor system: Consists of a closed tank, where the slurry is mixed using a mechanical mixer. The liquid slurry leaving the reactor is partly recycled, and the remaining flow is sent to other downstream slurry-bioreactor. The mixer design is critical to prevent settling of the soil in the bioreactor
5. Clarifier system: A suitable coagulant is added to the effluent of the final bioreactor, and a clarifier is used to separate the liquid from the treated soil and biomass. Part of the solid phase is recycled back to each bioreactor, to supply acclimated microorganisms and provide some dilution of the contaminated solid (soil) feed to each bioreactor. The liquid effluent is sent to a wastewater treatment plant

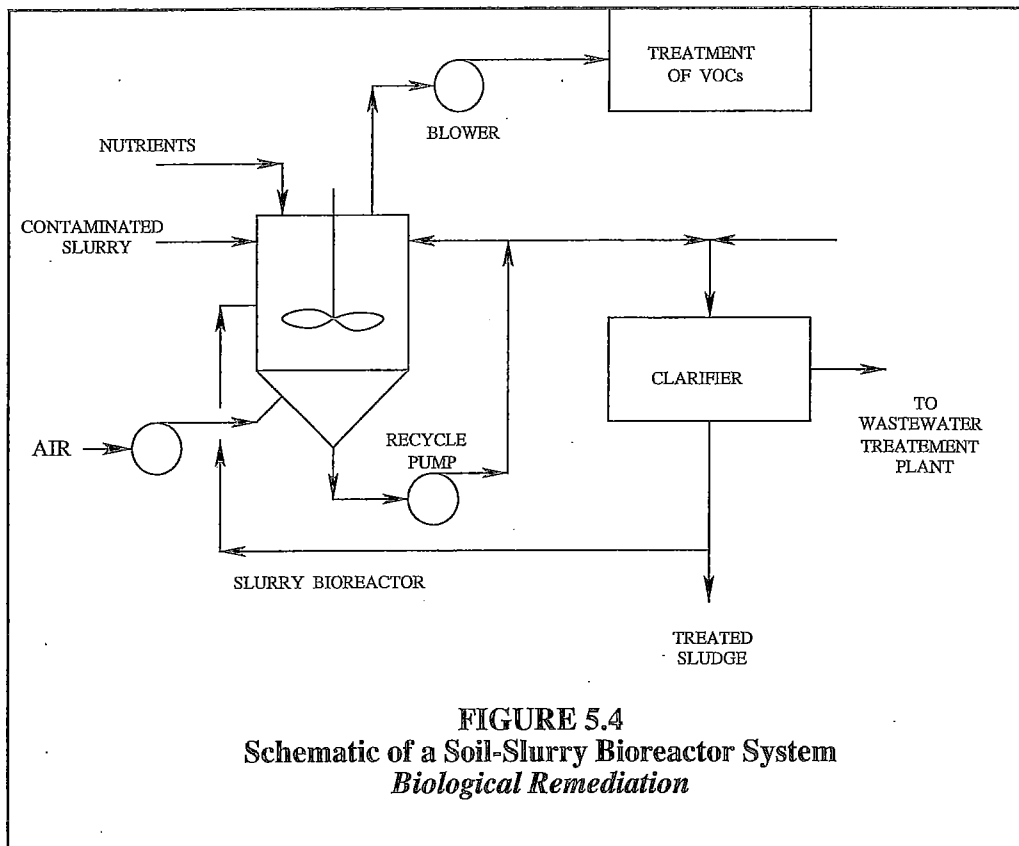


FIGURE 5.4
Schematic of a Soil-Slurry Bioreactor System
Biological Remediation

5.5.2.2. Factors Affecting Off-Site Treatment

The major factors affecting soil-slurry treatment are as follows:

- Solubility of the contaminant in water
 - One of the mechanisms for the biodegradation of the contaminant is its solubilization in the water and subsequent degradation by the microbial population in the water. The extent of the contaminant's solubilization in the water phase depends on its aqueous solubility

- Adsorption/Desorption characteristics of the contaminant
 - All contaminants exhibit to varying degrees adsorption on the surface of the soil matrix. These contaminants remain on the soil particles in the slurry reactor and hence can only be biodegraded by the microorganisms which are attached to the soil particle surface. Some contaminants have a lower affinity for the soil surface, and hence desorb in aqueous solution. The extent of biotreatment in a slurry reactor depends on the adsorption/ desorption characteristics of the contaminant
- Aging of the soil since the onset of contamination
 - When soil is contaminated by an organic contaminant, usually, the organic chemical does not diffuse into the interior of the soil micropores, but rather remains on the outside surface of the macropores. However, as time elapses, the organic contaminant diffuses slowly into the interior of the soil micropores, which is referred to as "soil aging." Furthermore, some chemicals also undergo slow chemical transformations, usually catalyzed by the inorganic constituents of the soil matrix, which leads to chemical binding of the chemical to the soil matrix
 - The soil aging process makes the chemical difficult to treat, since for biological degradation, the chemical has to diffuse out from the interior of the micropores, which is inherently a slow process, and depends on the diffusivity of the contaminant
- Concentration of the microbial population in the reactor
 - The microbial population in the reactor is maintained by proper addition of nutrients, oxygen, and maintaining pH and reactor temperature
- Screen out gravel and coarser sized particles
- Plasticity of soil to accommodate thorough mixing in reactor (minimal clumping)

5.5.2.3. Advantages/Disadvantages

The major advantages of soil-slurry treatment are as follows:

- Proper operating conditions for effective biotreatment can be achieved in the slurry reactors. This allows the achievement of specified (limits set by designer) contaminant exit concentrations, and effective monitoring of the biotreatment process
- Treatment costs using slurry bioreactors are over 50% less than alternative technologies, such as incineration
- Slurry treatment provides effective control of volatile emissions and eliminates some statutory concerns related to contaminant migration

The major disadvantages of soil-slurry treatment include:

- Excavation of the site and transportation of the soil, which is an expensive process
- Slurry treatment requires material handling equipment with possible contaminated dust generation, emission of volatile compounds etc. When volatile compounds are present, bioventing and vacuum extraction methods may have to be used as pretreatment before off-site soil-slurry treatment can be used
- The water decanted from the clarifier system has to be subsequently treated in a treatment plant
- Effective slurry reactor treatment requires that the chemical can be easily desorbed from the soil matrix and solubilized in the water phase. Furthermore, the chemical should biodegrade at reasonable rates, so that the size of the bioreactor is not large

- Requires energy consumption, for mixing and pumping of the slurry, pumping of air, etc.

5.5.2.4. Site Characterization Factors for Using Soil-Slurry Treatment

Usually slurry-reactor treatment is used for somewhat water soluble contaminants. Soil-slurry biotreatment can be used if the contaminated soil can be excavated, when the contamination is only present in surface soil, and the extent of contamination is small.

5.5.2.5. Approximate Costs

Treatment costs using slurry reactors range from \$100 to \$150/cubic yard. Costs ranging from \$125 to \$160/cubic yard are incurred when the slurry-bioreactor off gas has to be further treated, due to the presence of volatile compounds.

5.5.2.6. Additional Issues and Research Needs

Additional issues and research needs are as follows:

- Effect of soil aging on the effectiveness of slurry-reactor treatment
- Development of more efficient soil/slurry agitation methods.
- Adsorption/desorption kinetics of multiple contaminants from the soil matrix
- Biodegradation kinetics for strongly adsorbed contaminants
- Effect of soil properties, such as particle size, pore size distribution, etc. on biodegradation kinetics
- Use of other bioreactor designs, such as plug flow, as compared to completely-mixed systems or combination of reactor systems

5.5.3. In Situ Bioremediation

5.5.3.1. Overview

Biodegradation finds wide applicability for in-situ bioremediation. Low capital and treatment cost can make it preferable over other *ex situ* treatment technologies. As an *in situ* treatment, bioremediation is particularly advantageous when a contaminant cannot be pumped out from an aquifer. The technology, however, may not be applicable in all the situations. *In situ* applications are limited to environments where conditions are amenable to easy dispersion of oxygen and nutrients, as well as injected microbes. In-situ bioremediation is also better suited for shallow contamination.

As mentioned above, it is a case specific technology and its applicability depends on: 12, 13, 14, 15

- Contaminant characteristics
- Microbial requirements
- Hydrogeology of the site

The following parameters affect the applicability of *in situ* bioremediation and require further discussion:

- Biodegradability
- Inducibility and stimulation
- Feasibility in combination with other technologies
- Contaminant characteristics

5.5.3.2. Biodegradability

In order to employ *in situ* biodegradation as a remediation technology, the first step is to investigate the biodegradability of the compound of interest. Based on bench and pilot-scale tests with indigenous soils and contaminants, it may be ascertained whether the contaminant is biodegradable by indigenous microbes or special microbes are required. A compound could be easily biodegradable resulting in complete mineralization; it could be persistent requiring specific conditions; or it could be recalcitrant indicating no degradation at all. In some cases, as explained below, biostimulation and biotransformation of the contaminant can result in efficient disposal of the compound.

5.5.3.3. Inducibility and Stimulation

Once the biodegradability of the compound is established the next step is to find the optimal conditions for the process.

The growth requirements of the microorganisms must be considered, such as:

- Is TEA required?
- What nutrients are required?
- What is the favorable temperature range?
- What is the appropriate pH range?

In situ bioremediation may require:

- Pumping the microorganisms into the sub-surface
 - Especially if the sub-surface environment does not have an enriched population of the microbes
- Adding nutrients
 - Oxygen or nitrate, depending on the terminal electron acceptor
- Adding co-substrates
 - If co-metabolism is necessary to induce biodegradation
- Selecting a species appropriate to a site's temperature and pH

Stimulation of appropriate microorganisms can cause biodegradation of ordinarily non-degradable, or recalcitrant, compounds under given site conditions. The microbial population created can be used directly, or indirectly, to biodegrade the compound as a result of co-metabolism or biotransformation.

Example: Chlorinated aliphatic compounds, for example have been transformed to stable nontoxic products through a co-metabolic process by

stimulating growth of indigenous methane oxidizing bacteria (methanotrophs) at the site.

Microbial activity in an environment can be greatly influenced by the site characteristics. Groundwater, for example, shows a higher microbial population and a significantly greater utilization of the dissolved organics by the bacteria than in the case of stream water.

If a site does not have a good microbial population, it can be induced artificially by "seeding" the site with the desired strains of the microorganisms.

This process is advantageous in providing:

- The option to choose an appropriate microorganism
- The possibility of engineering the microorganism being induced

These advantages, however, are greatly off-set by:

- The difficulty of the microorganisms to adapt and survive in the new environment
- The failure of technology to transfer from laboratory conditions to field conditions
- The unpredictability of survival of induced populations due to extraneous factors in the environment, such as:
 - Competition with existing species
 - Effects of metabolically and co-metabolically degraded substrates on the induced and existing populations
 - Possible interactions and products of biodegradation
- The inability to predict the rate of degradation from lab results due to inconsistency with conditions
 - No constant can be set, so lab results should be considered only as rough estimates

5.5.3.4. Feasibility in Combination with Other Technologies

In situ biodegradation can be enhanced through its combination with other technologies appropriate to the characteristics of the site and the contaminant. Approaches being reviewed for combination with biodegradation are ¹⁶.

- Ozonation
- Hydrogen peroxide treatment
- Genetic engineering
- Vapor gas extraction (bioventing)

5.5.3.5. Factors Affecting *In Situ* Bioremediation

The major factors that affect *in situ* bioremediation are as follows:

- Availability of oxygen. Oxygen diffusion through the soil can become rate limiting, especially in the subsurface environments
- Availability of nutrients. Nitrogen and phosphorous are required for microbial growth. Nutrients may be present in the soil matrix, or may have to be added externally

- pH of the soil matrix. pH control is necessary to support microbial growth, and hence *in situ* bioremediation in low pH soils is difficult
- Temperature. Microbial growth rate is affected by temperature. As temperature decreases, microbial growth slows down considerably
- Nature of the contaminant. The biodegradability of the contaminant depends on its chemical structure, i.e., size of carbon backbone, presence of functional groups, etc.
- Heterogeneous subsurface conditions limiting thorough dispersion of microbes, nutrients, and oxygen.

5.5.3.6. Advantages/ Disadvantages

The major advantages of *in situ* bioremediation are as follows:

- Lower capital cost for implementation
- Does not require excavation and transportation of the soil
- Does not cause cross-media contamination, since the soil is not disturbed
- Can be used for bioremediation of large-scale contamination of subsurface soils

The major disadvantages of *in situ* bioremediation are as follows:

- Difficult to monitor and quantify the extent of bioremediation
- Can produce biodegradation products, which may remain at the site
- Can take a very long time, depending on the type of contamination, and environmental conditions (thus involving considerable operation and maintenance costs)
- Requires favorable environmental conditions, such as temperature, moisture, pH of the soil matrix, etc.
- May be required to be combined with containment technology to avoid leaching and further contamination spread while remediation is in progress.

5.5.3.7. Site Characterization Factors for Selection of Techniques

Accessibility of the site can be important in determining feasibility of bioremediation. Typical site conditions that are important are:

- Location of contaminant
- Availability of land
- Building or utility locations
- Local population

Location of contaminant is important in determining whether nutrients, TEA, microbes etc. can be applied to the contaminated portion of the site. Biodegradation of a chemical depends on the spatial characteristics of the site. If the site characteristics vary with position, the biodegradation characteristics of the contaminant would also vary. A study was done to evaluate the effect of spatial variation of the contaminant on biodegradation at three sites representing a range of environments from fresh water to estuarine to marine systems. The heterotrophic rates of uptake at these sites differed, with the highest rate at the fresh water site and the lowest rate at the marine site. These rates were also a function of water temperature and this seemed to affect the fresh

water and estuarine environments more than the marine environment. Thus, it may be inferred from this study that biodegradation rates are a function of both time and spatial distribution of the substrate.

Land area or building location may prohibit the building of a bioremediation treatment system, such as bioreactors, aboveground piles, or distribution systems for air/water for in-situ bioremediation. Finally, political and other issues concerning the local population may render bioremediation impractical. Local perceptions may be very important element in determining whether bioremediation will be an acceptable treatment process¹². Bioremediation has been accepted by EPA on a number of Superfund sites. At Superfund sites selection criteria partly depends on public acceptance of the alternative.

The surface area and depth of contamination at the site are important factors for in-situ bioremediation. Other factors are soil structure and site geology. Permeability of the soil phase is critical for application of in-situ bioremediation. Low permeabilities generally indicates that the mass transfer of both TEA and nutrients may be severely impeded, making aboveground bioremediation more applicable. Other geological consideration include groundwater level and flow. Soil pH affects sorption of ionizable compounds in addition to limiting the type of suitable microorganisms. Temperature has a major impact on bioremediation rates. Concentrations of iron and manganese are important because high concentrations of these metals can cause precipitation under aerobic conditions due to infiltration of oxygen. Other heavy metals are important because at toxic levels they can inhibit the activity of microorganisms.

5.5.3.7.1. Hydrogeology (Saturated Soil)

Aspects of hydrogeology which affect biodegradation are:

- Specific yield and transmissivity
- Depth and thickness of the contaminated aquifer
- Contaminated aquifer heterogeneity
- Direction of ground water flow
- Recharge of contaminated areas
- Seasonal fluctuations of water table

The site should have the following characteristics:

- The conductivity of the soil should be high enough to allow addition of the nutrients, microorganisms etc.
 - Should be higher than 10^{-4} cm/s
- The heterogeneity of the subsurface should be limited, so that , irregularities can be accommodated in plans for distribution of microbes and nutrients.
 - For example, model should account for variations in hydraulic conductivity

5.5.3.7.1.1. Soil Properties (Vadose and Saturated Zone)

Soil properties such as water content, organic carbon content, and porosity affect the rate of biodegradation. If, for example, a soil has high porosity and low water content it will favor diffusion of oxygen, thus promoting aerobic reactions. A high organic carbon content of the soil may provide a carbon and energy source to the microorganisms and in some instances can result in

co-metabolic degradation of the contaminant. Soil particles also provide surfaces for bacterial attachment and play an important role in microbial degradation. Studies show that the rate of biodegradation is directly proportional to the exposed surface area for the microbial attachment.

5.5.3.7.1.2. Groundwater Quality

5.5.3.7.1.2.1. Inorganic Nutrient Levels

Nutrient levels must be sufficient and constant for an efficient and uninterrupted process. If the site is not sufficiently rich in nutrients, it should be pumped periodically depending on the specific problem. Dispersion is the main mechanism for spreading the nutrients in groundwater. It is essential to restrict the area of the plume to avoid excess spreading of the plume resulting in wastage of nutrients. Interceptor walls, trenches, and extraction wells are commonly used to contain a plume.

5.5.3.7.1.2.2. Precipitation of Inorganic Nutrients

Compatibility between the site geo-chemical characteristics and the nutrient solution being pumped inside is very important. If the nutrients precipitate inside the soil, it could block the pores thus cutting off both water and oxygen supply. Other soil chemistry factors that are important are pH, and organic content.

5.5.3.7.1.2.3. Dissolved Oxygen Content

Dissolved oxygen in the sub-surface is the most important rate limiting factor for aerobic biodegradation. For a rapid and steady biological reaction, a constant and uniform supply of dissolved oxygen is essential. Oxygen can be supplied in the form of:

- Air
- Pure oxygen
- Hydrogen- peroxide

Use of Hydrogen-peroxide can create some problems:

- Is toxic to some of the microbial species
- Should be introduced at the level of 50 ppm and gradually increased to the required level (should be around 750-1000 ppm)
- Is very unstable and degases easily to water vapor and oxygen; therefore care must be taken while using it.

5.5.3.7.1.2.4. Presence of Other Terminal Electron Acceptors.

Some microbes do not require oxygen as the terminal electron acceptor (TEA). In such cases it should be ensured that the other required TEA is in sufficient quantity at the site. Nitrate is a common terminal acceptor used by some microbes. It is pumped into the ground when not available in sufficient quantities.

Adding Nitrate:

- Offers a cost effective technology
- Relieves the problem of solubility
- Raises problems as a drinking water contaminant

5.5.3.8. Approximate Costs

The cost of treatment depends on the nature of the contaminability (the solubility, volatility, and sorptive ability of the contaminant), the location and extent of contamination, the hydrogeology of the site, and the goal of the remediation project, i.e., source remediation or plume control.

The cost of bioremediation project varies with the methodologies used and on the location, quantity, and characteristics of the wastes present. If groundwater is impacted, an average of \$100 is spent for each gallon of gasoline spilled, according to a 1988 study by the American Petroleum Institute.

Bioremediation of contaminated soils is inexpensive compared to some of the non-biological methods, such as thermal treatment. Conventional land treatment costs range from \$50 to \$80/cubic yard.

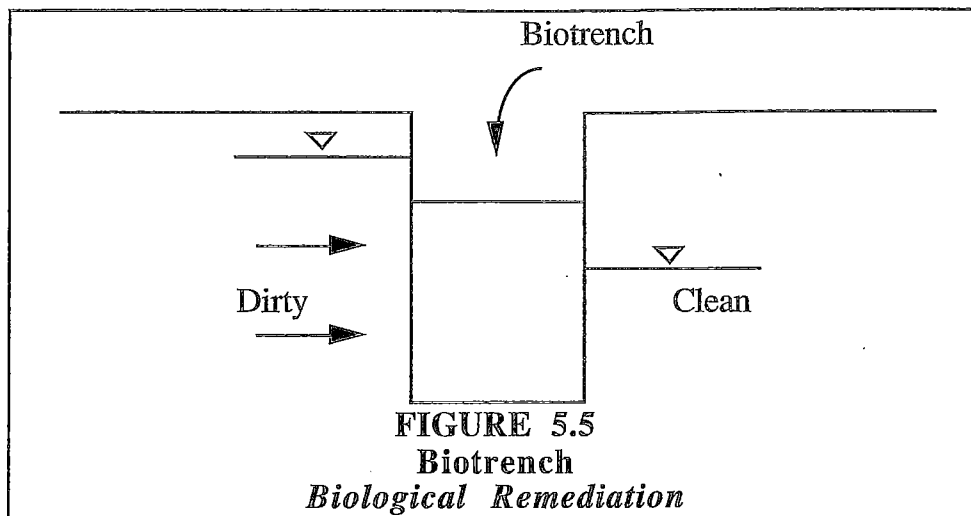
Bioremediation of fuel contaminated groundwater is cost effective, but the process is often unable to achieve the low effluent concentrations achieved by other methods. An *in situ* bioremediation scheme can cost \$8 to \$15 per pound of contaminant \$66 - \$123/cubic yard of material treated. Since the cost of bioremediation is mostly site specific these reported values are an over simplification, and are only provided as a point of reference to compare other treatment technology with.

5.5.3.9. Additional Issues and Research Needs

In light of the potential of bioremediation process to eliminate contaminants without causing cross-media pollution and its over all cost effectiveness, there is a growing need for further and research in this area ^{13, 15, 17} There are several outstanding issues that need to be addressed and research needs to be supported, especially since an expanding requirement for bioremediation technologies can be seen in the future. Some of the issues and research needs have been listed below:

- Quantitative determination of *in situ* biodegradation rates
- Determining biodegradation kinetics for complex mixtures of chemicals, rather than for single chemicals
- Benefits of seeding contaminated sites with acclimated microorganisms
- Role of indigenous microorganisms in effective bioremediation and quantifying the impact of stimulating biological activity by addition of nutrients, water, oxygen, etc.
- Transport of contaminants and microorganisms in soil environments

A biotrench is a technology that involves developing a trench down gradient of the limits of the contaminant plume. The trench contains microbes fed by nutrients in the waste. As water flows through the trench microbes act on the contaminants and "clean water" flows out on the downgradient side (see Figure 5.5).



5.5.4. Bioventing

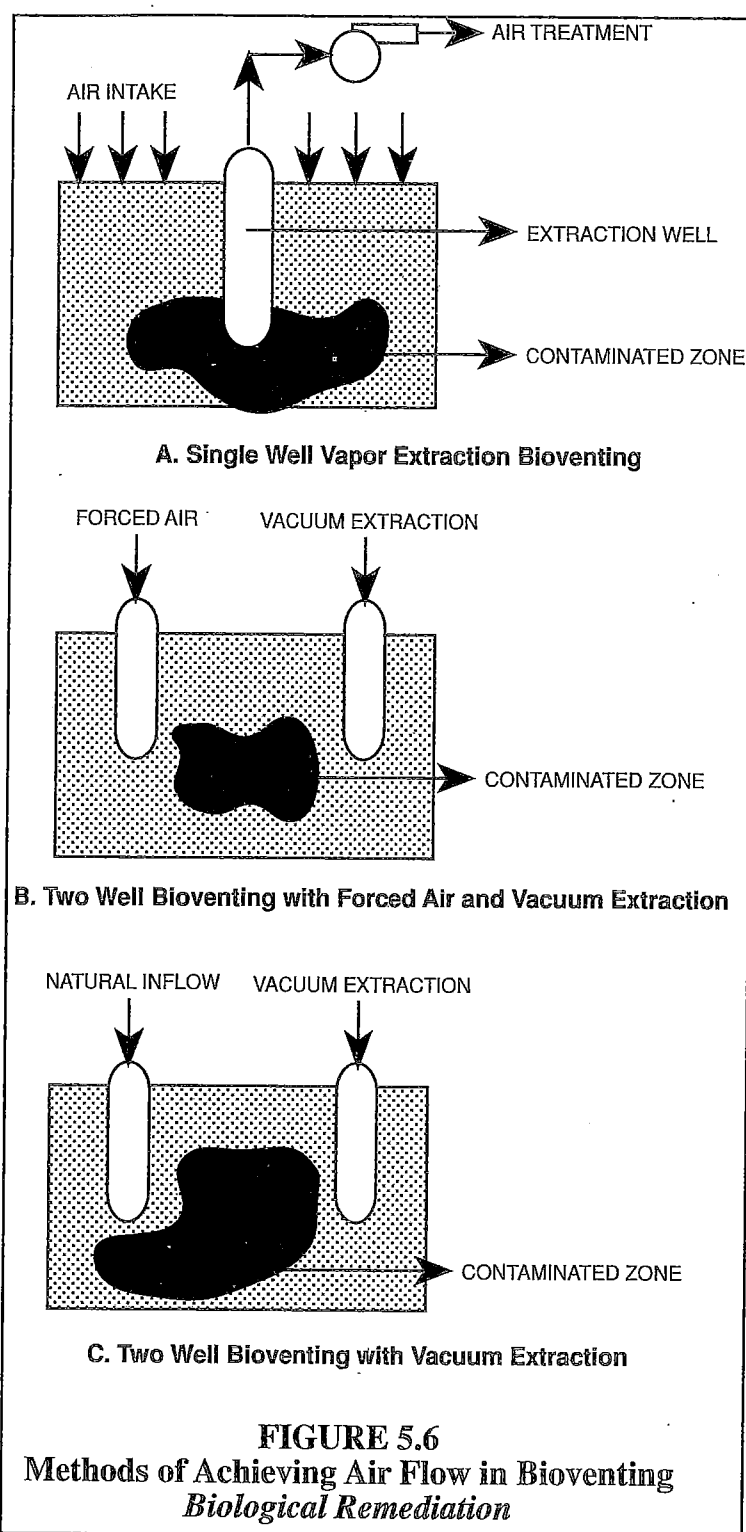
5.5.4.1. Feasibility and Conceptual Approach

Bioventing uses air movement to provide oxygen for aerobic degradation using either indigenous or introduced microorganisms. While some organics will be present in the exiting air streams, in-situ biodegradation is the main goal, and occurs mainly for less volatile materials, such as diesel fuel. For bioventing to work, sufficient water must be present in the unsaturated zone. In addition, nutrients, such as orthophosphate and ammonium nitrogen may need to be introduced to achieve optimal degradation rates^{13, 15, 17}.

There are three ways of achieving air flow in bioventing¹⁴.

- Single well vapor extraction: Vacuum extraction is applied to one well causing ambient air to enter into the surrounding soil (see Figure 5.6 a)
- Two wells are used with air pumped into one well and extracted from the other well (see Figure 5.6.b.)
- Two wells are used with vacuum extraction applied to one well causing air to enter into the soil primarily through the other well (see Figure 5.6.c)

The level of vacuum that can be applied in the extraction well can vary from high vacuum (100 inches Hg) to low vacuum (20-30 inches Hg).



5.5.4.2. Factors Affecting Bioventing

Factors affecting bioventing can be classified into two categories^{18, 19, 20, 21}:

- Substrate characteristics
- Environmental factors

Substrate limiting factors are as follows:

Chemical contaminant(s) must be volatile and biodegradable. Furthermore, since bioventing is based on air flow, a soluble contaminant will reside in the soil moisture to a greater extent than chemicals with low solubility. For maximum effectiveness of bioventing, compounds with high volatility (high vapor pressure) and high biodegradability are optimal. For many contaminants, volatility and biodegradability are synergistic since the compounds that are not volatile biodegrade easily. This is especially true for hydrocarbon fuels wherein light products are treated primarily by volatilization and heavy products by biodegradation.

The major problem is caused by high solubility, as in the case of Methyl ethyl ketone (MEK). Bioventing for MEK is not very efficient due to its high solubility in soil moisture, which slows the process of mass transfer from the adsorbed phase to the gas phase. For most effective bioventing, the mass transport has to occur from the adsorbed solid phase to the gas phase, which depends on the compound's vapor pressure. For soluble compounds, the mass transport occurs from the solid phase to the liquid phase and then to the gas phase, which then becomes dependent on the Henry's Law constant and solubility. The major factor that offsets the negative impact of solubility is that in bioventing, air is supplied which promotes the rate of biodegradation. Hence, the decrease in volatilization due to solubility is offset by an increase in biodegradation.

Environment limiting factors are as follows:

- (1) Permeability of the soil is very critical for forced air circulation through the soil matrix. The flux of air flow through a soil matrix can be expressed using Darcy's Law:

$$F = \left(\frac{DBo}{v} \right) \frac{\partial P}{\partial x}$$

where:

- F = flux
v = gas viscosity
Bo = soil permeability
D = gas density
 $\partial P / \partial x$ = pressure gradient

Generally, significant decrease in flux occurs when the permeability is less than 10^{-4} cm/sec. At this point, the flux drops exponentially with decreasing permeability.

- (2) The second major environmental factor in bioventing is the heterogeneity of the soil matrix. Generally, a soil matrix consists of high permeability sections interspersed between low permeability areas. Sometimes contamination can spread to low permeable areas through high permeability soil formations. The key is to access the high permeability formations by suitably locating trenches and vacuum wells.

(3) The third important factor is the degree of water saturation, since air flow rate is proportional to air filled soil porosity. Soil moisture contents approaching 100% saturation, reduce the amount of air filled porosity, which lowers the effectiveness of bioventing. When contamination occurs below the water table, either the water must be removed by dewatering, or air must be sparged into the water.

(4) Other environment limiting factors include temperature, pH, and availability of nutrients

5.5.4.3. Advantages/Disadvantages

The major advantages of bioventing are as follows ^{22, 23}:

(1) Allows direct extraction of organic compounds and enhances biodegradation. A bioventing system is capable of removing hundreds to thousands of pounds per day of highly volatile organic compounds. Table 5.3 gives calculated mass removal (lbs/day) rates for a compound with an average molecular weight of 100 and air flow of 100 SCFM.

Table 5.3
Estimate of mass removal rates of some volatile compounds

level of vacuum	Contaminant Removal Rates (lbs/day)		
	100% of vapor pressure	10% of vapor pressure	1% of vapor pressure
0-15mm Hg (Example Toluene)	0-400	0-20	0-4
15-30 mm Hg (Example Benzene)	400-2500	20-120	4-10
30-45 mm Hg	2500-5000	120-500	10-75
45-60 mm Hg	5000-7000	500-800	75-100
60-90 mm Hg (Example Acetone)	7000-10,000	800-1000	100-120

% of vapor pressure depends on level of vacuum created during bioventing using vacuum extraction.

Enhanced oxygenation due to air flow can also cause significant increases in biodegradation. Table 5.4 gives values for oxygen supply (lbs/day) depending on air flow rate and percent utilization of the oxygen supplied.

Table 5.4
Oxygen Supply Rates

Air flow rate (SCFM)	Percentage utilization		
	100 (lbs-O ₂ /day)	10 (lbs-O ₂ /day)	1 (lbs-O ₂ /day)
25	800	90	8
75	5000	500	60
100	7000	800	80
150	10,000	1000	100

As can be seen from the above table, even a small air flowrate of about 25 SCFM can provide significant amounts of oxygen, which even at low utilization rates can provide substantial enhancement of biodegradation activity.

(2) Bioventing is a relatively inexpensive process for in-situ bioremediation, provided, that no air vented treatment is required. Table 5.5 gives the cost of supplying oxygen (\$/day) by vapor extraction in bioventing as compared to other methods such as peroxide addition and aeration ^{19, 24}.

Table 5.5
Cost Estimates for Supplying Oxygen (\$/day)

Type of Technology	Percent utilization		
	100	10	1
Peroxide	80	200	800
Aeration	100	200	300
Bioventing using vapor extraction	0.8	3.0	25

It is assumed that peroxide is added at 2000 ppm concentration at 10 GPM flowrate. Aeration is achieved by injection of aerated water (dissolved oxygen 10 ppm) at 20 GPM flow rate or by sparging in wells. It is assumed that in bioventing the air flowrate is 100 SCFM and hence no subsequent vapor treatment is needed.

(3) Minimal exposure of the public (or the contractors) to the affected zone. This is a basic advantage of all in-situ processes which results in minimal disruption of surface level activities (operation of airports, plants, etc.) once the process installation is completed. In-situ treatment, such as bioventing can proceed while normal site functions continue.

The major disadvantages of bioventing are as follows:

(1) A minimum permeability value of 10^{-5} cm/sec is needed for achieving effective bioventing. The limitations on permeability depends on the type of system being used for achieving air flow. The types of systems that are possible are as follows:

- High vacuum (20-30) inches Hg versus low vacuum (about 100 inches Hg)
- Single or multiple wells with forced pumping of air (Most venting applications end up with wells at 10-20 ft. spacing. Single or dual wells are only utilized for small contaminated areas.)

The main advantages of high vacuum are greater radius of influence, shorter remediation times, and ability to treat lower permeable formations. Low vacuum systems are lower in cost, have lower maintenance costs, and allows for more uniform treatment. The main disadvantages of high vacuum bioventing are: significant waste upwelling causing well fouling; increase a channeling of the air flow; and higher air velocities resulting in increased frictional losses.

In single well vapor extraction, for homogeneous soils the radius of influence is theoretically symmetrical around the well and hence a proper distribution of wells is required in the contaminated area.

Using two wells with air being forced into one well results in better air distribution, but limits the area of influence primarily to the area between the two wells and can force the contaminant away

form the receiving well, if the injection well is located outside of the capture zone of the extraction well.

(2) Bioventing systems suffer from "aging" as the contaminant region decreases. This aging is primarily caused by diffusive flow rather than advective flow in the contaminated region. Usually this problem can be reduced by changing the configuration of the extraction wells thereby changing the air flow pattern. Changes in air distribution need to be optimized for effective bioventing throughout the treatment period.

(3) One potential disadvantage of an in-situ process, such as bioventing, is that contaminant materials are mobilized by the movement of air and water around the contaminated zone. This requires close monitoring of air and water around the contaminated zone to ensure that the contaminant does not escape the air and water capture systems. Flow models used to describe contaminate transport should be calibrated with actual site data so that the fate of the contaminant can be determined with a certain degree of confidence.

(4) Due to variations in site geology and contaminant distribution, the treatment time cannot be guaranteed, in spite of laboratory and pilot tests. Usually there are regions where the concentration remains high (hot spots) as compared to the treatment standard.

(5) Due to the fact that bioventing is a relatively new technology, public acceptance of the technology may require detailed explanations and meetings. Although bioventing has a history of full-scale success in cleaning sites, public acceptance is not guaranteed. The practitioner needs sufficient experience base to apply bioventing successfully.

5.5.4.4. Design Considerations of Bioventing

The design of the bioventing system requires proper specification of pumps and piping. The driving force for the creation of a vacuum in the soil is a positive displacement blower, a centrifugal blower, or a vacuum pump, depending on the type of system used as shown in Figure 5.6. Factors that determine blower design are level of vacuum, areal extent of contamination, air permeability of soil, and pressure losses through the pump, piping and collection system. Due to safety considerations, sites where explosive gases are present, explosion-proof housing for motors and liquid sealed vacuum pumps with minimal spark potential have to be used.

The piping used to connect the wells to the blower and/or vacuum pump is simple for single well systems but becomes increasingly complicated as more injection and extraction wells are used. Usually the piping is 6 inches in diameter and material of construction can be polyvinylchloride (PVC) (Schedule 40 or 80), polypropylene, high density polypropylene, or stainless steel. The piping can be above ground or buried underground for sites in active use. In colder climates the piping has to be insulated to prevent condensed water from freezing. Vapors exiting the extraction wells are connected to knock-out drums to separate fine silt particles and then cooled to condense moisture. If a high vacuum is used which can cause significant increases in the water table level, large quantities of water are removed and in cases where this water is contaminated, suitable water treatment systems have to be used. In addition there are, problems with excess moisture in the vapors expensive condensation treatment may be required.

5.5.5. Site Characterization

The surface area and depth of the contaminated site is important for evaluating bioventing costs, in addition to time for achieving cleanup goals also critical. Capital cost are low, but operation and maintenance can be very high for extended operations. Traditionally, site areas have varied between 20-75,000 m² in the 3-10 m depth range. Sites where bioventing has been applied can be

categorized into two classes: gas filling stations, where the surface areas typically varied between 400-1000 m², and large chemical industry (refinery) sites, where the areas of contamination may be as large as 20,000-75,000 m².

Bioventing is typically applied to sandy subsurface conditions, although some application sites have included occasional clay lens. Only in exceptional cases, has bioventing been applied at sites with extensive overburden clay or fractured bedrock. Permeability is an important factor in determining feasibility of bioventing, and for the projects reviewed, permeability varied between 10⁻² to 10⁻⁸ m/s, with average value of 10⁻³ m/s. Generally, a value of 10⁻⁵ cm/s is regarded as the minimum permeability for successful application of bioventing. Soil pH also affects sorption of ionizable compounds in addition to limiting the types of microorganisms in the subsurface. Methanogens, which are responsible for mineralization of some aromatic compounds, are inhibited at pH values below 6. Biodegradation of many organic pollutants may be limited by insufficient concentrations of oxygen or unfavorable redox conditions. Temperature also influences microbial metabolism, and usually the temperature varies seasonally up to a depth of 10 m. The impact of temperature is most pronounced up to the frost line (typically up to a depth of 2 m). Determination of iron and manganese concentrations is important because high concentrations of these metals can cause precipitation under aerobic conditions. Other heavy metals can be important, because at toxic levels they can inhibit the activity of microorganisms. Inorganic nutrients like nitrogen and phosphorous may be limiting when the carbon/nitrogen/phosphorous (C:N:P) ratio is unfavorable. Determination of ammonium, nitrate and nitrite levels is important before applying bioventing.

Site characterization involves determining the microbiological, hydrogeological, and chemical/physical parameters. Preliminary biotreatment studies at the laboratory scale are needed to determine the optimal stimulatory conditions for effective biodegradation at a given site.

5.5.6. Approximate Costs

Costs for bioventing depend on several factors:

- Geology and soil structure
- Type and concentrations of contamination
- Distribution of contamination in the soil
- Total surface and volume of contamination
- System characteristics of the bioventing process: type of air circulation, level of vacuum, number of wells etc.
- Time of application

Generally one-third of the total cost is for preliminary research and installation costs. The remaining two-thirds of the total costs are spent on operation and maintenance costs.

For a one acre site contaminated with gasoline with a treatment period of 1.5-2 years, the cost of bioventing is approximately \$250,000 without vapor treatment and \$350,000-\$400,000 with the vapor treatment. Enhancement of biodegradation by adding nutrients lowers the cost of vapor treatment. However, nutrient addition can add \$20-40,000 to the total cost.

6. HEALTH AND REGULATORY ISSUES

6.1. Health and Safety Issues

The two main public health and safety questions that determine the feasibility of bioremediation are:

- Can the hazard posed by the contaminant be removed in a timely manner, and can the hazard be contained during treatment. This may reflect in additional treatment cost which may be balanced against other more rapid treatment.
- Does the selected bioremediation process pose any hazards, such as toxic by-products, fire or explosion hazards? Other hazards include dust and vapors generated by land farming activities.

Information regarding health and safety issues can be obtained from risk assessments and the development of a health and safety plan for the project.

6.2. Regulatory Issues

Local, state, and federal regulations that are applicable need to be considered. For example, Land Disposal Restriction (LDR) regulations may prevent the use of certain bioremediation technologies, such as above ground biopiles, while in-situ treatment may be allowed. LDR's would not affect above-ground biopiles unless the waste was to be permanently disposed in the biopile. However, if contaminated soil was classified as hazardous under RCRA, one would need a RCRA treatment/storage permit for the waste pile, which can be a lengthy and expensive process requiring significant closure monitoring. Although, an in-situ treatment operation would not require a RCRA permit.

APPENDIX

1. BASIC MECHANISM OF BIODEGRADATION

As explained above all microbes require a carbon and energy source for their growth.

Types of carbon include:

- Organic (wide variety available)
- Inorganic (e.g., dissolved carbon dioxide)

Types of energy include:

- Light energy such as sunlight
- Chemical reactions
 - The amount of energy available from these sources depends not only on the energy source but also on the terminal electron acceptor used.

Most of the energy in biological reactions is obtained from electron pair transfers (oxidative phosphorylation) i.e., electrons are transferred from one compound to the other in the order of decreasing energy level. Each time these electrons are transferred a small amount of energy is generated which is either stored in the form

of ATP - the energy currency of a cell, or used up for cell functions. These electrons are donated by the parent food molecule. They pass through a number of intermediate molecules releasing energy in every transaction they pass through and are finally accepted by a terminal electron acceptor (e.g., oxygen is the terminal electron acceptor for aerobes) which gets reduced and does not transfer the electrons further down—thus stopping the reaction. Availability of this terminal electron acceptor is essential for the constant energy supply required by the cells.

Reference may be made here to the "electron tower", which is a table showing various electron acceptors in the order of decreasing energy levels (App. Table 1.1). For a biological reaction to be energetically favorable i.e. in order to have $\Delta G < 0$, the energy source must be positioned above the terminal electron acceptor in the energy tower. The farther apart the two are, the greater is the energy yield per molecule of substrate oxidized. The spontaneity of a biological reaction, in the way it is written, can be quantified by the Nerest Equation:

$$\Delta G = -nF\Delta E$$

Where:

ΔG = Free energy change of the reaction. If ΔG is < 0 the reaction is spontaneous. If ΔG is > 0 then energy is required for the reaction to proceed.

n = Moles of electrons transferred per mole of reactant/substrate consumed.

F = Faraday's Constant = 25,000 cal/volt

ΔE = Change in redox potential (volts)

Oxygen is placed very low in this tower and this is the reason that more energy is yielded using oxygen as the terminal electron acceptor than most other terminal electron acceptors. This also explains why facultative bacteria yield more energy aerobically than that in absence of air.

App. Table 1.1
Reduction Potentials of Some Redox Pairs of Biochemical and Microbiological Importance

Redox Pair	E'°(V)
SO ₄ ²⁻ /HSO ₃ ⁻	-0.52
CO ₂ /formate	-0.43
H ⁺ /H ₂	-0.41
Ferredoxin ox/red	-0.39
Flavodoxin ox/red*	-0.37
S ₂ O ₃ ²⁻ /HS ⁻ + HSO ₃ ⁻	-0.36
NAD/NADH	-0.32
Cytochrome c ₃ ox/red	-0.29
CO ₂ /acetate ⁻	-0.29
S ₀ /HS ⁻	-0.25
SO ₄ ²⁻ /HS ⁻	-0.25
CO ₂ /CH ₄	-0.24
FAD/FADH	-0.22
HSO ₃ ⁻ /S ₃ O ₆ ²⁻	-0.20
Acetaldehyde/ethanol	-0.20
Pyruvate ⁻ /lactate ⁻	-0.19
FMN/FMNH	-0.19
Dihydroxyacetone phosphate/ glycerolphosphate	-0.19
Flavodoxin ox/red	-0.12
HSO ₃ ⁻ /HS ⁻	-0.11
Menaquinone ox/red*	-0.075
APS/AMP + HSO ₃ ⁻	-0.060
Rubredoxin ox/red	-0.057d
Acrylyl-CoA/propinyl-CoA	-0.015
Glycine/acetate ⁻ + NH ₄ ⁺	-0.010
S ₄ O ₆ ²⁻ /S ₂ O ₃ ²⁻	+0.025
Furmate/succinate	+0.030
Cytochrome b ox/red	+0.030
Ubiquinone ox/red	+0.11
Cytochrome c ₁ ox/red	+0.23
S ₄ O ₆ ²⁻ /S ₂ O ₃ ²⁻ + HSO ₃ ⁻	+0.23
NO ₂ ⁻ /NO	+0.36
Cytochrome a ₃ ox/red	+0.385
NO ₃ ⁻ /NO ₂ ⁻	+0.43
Fe ₃ ⁺ /Fe ₂ ⁺	+0.77
O ₂ /H ₂ O	+0.82
NO/N ₂ O	+1.18
N ₂ O/N ₂	+1.36

*Separate potentials are given for each electron transfer in this potentially two-electron transfer.

Cell yield - quantifies the amount of cells synthesized and is defined as the mass of microbial cells produced per unit mass of substrate consumed

Y = grams of cell mass/ grams of substrate consumed

2. AEROBIC BIODEGRADATION

2.1 Types of Microorganism

2.1.1 Methanotrophs and methylotrophs

Methanotrophs can oxidize methane and some other carbon compounds utilizing them as sole carbon and energy source. Organisms that can grow using only one - carbon compounds are generally called methylotrophs.

Presence of C1 metabolizers in the environment is of prime importance. As illustrated below, methanotrophs and methylotrophs play a key role in the degradation or initiation of degradation of a vast category of compounds. App. Table 2.1 lists some of the important species of these microorganisms and respective growth substrates.

App. Table 2.1
Classification of C₁ - Utilizing Microorganisms

Group	Examples	Growth substrates or C ₁ compounds metabolized
BACTERIA		
Obligate methylotrophs (Type I methanotrophs)	<i>Methylobacter</i> <i>Methylococcus</i> <i>Methylomonas</i> (<i>Pseudomonas methanica</i>) <i>Methylocystis</i>	methane, methanol, carbon monoxide (oxidized only)
Obligate methylotrophs (Type II methanotrophs)	<i>Methylosinus</i> (<i>Methanomonas methanooxidans</i>)	
Facultative methanotroph (similar to Type II obligate methylotroph)	<i>Methylobacterium organophilum</i>	methane, methanol, variety of heterotrophic substrates
Obligate methylotrophs unable to utilize methane	Bacterium 4B6	methylamine, di- and tri-methylamine
	<i>Methylomonas</i> M15	
	<i>Methylophilus methylotrophus</i>	methanol
	<i>Methylophilus methylotrophus</i> Organism W1	methanol, methylamine

App. Table 2.1
Classification of C1 - Utilizing Microorganisms (continued...)

Facultative methylotrophs (All grow on some heterotrophic substrates)	<i>Bacillus</i> PM6	methylamine, di- and tri- methylamine, tetramethyl- ammonium and trimethyl- sulfonium compounds methanol, formate, methylamine di- and tri- methylamine, tri- methylamine-N-oxide methanol, formate, methylamine, tri- methylamine, formamide, urea methanol, formate, methylamine, trimethylamine formamide formate, methylamine, di- and trimethylamine, tri- methylamine-N-oxide methylamine methylamine, di- and tri- methylamine, trimethyl- sulfonium compounds methanol, formate, methylamine methanol, formate, methylamine, di- and tri- methylamine, tri- methylamine-N-oxide
	<i>Bacterium</i> 5H2	
	<i>Bacterium</i> 5B1	
	<i>Hyphomicrobium</i> sp.	
	<i>Pseudomonas</i> AM1	
	<i>Pseudomonas</i> SL4	
	<i>Pseudomonas aminovorans</i>	
	<i>Pseudomonas</i> MA	
	<i>Pseudomonas</i> MS	
	<i>Pseudomonas</i> M27	
	<i>Pseudomonas</i> 3A2	
Autotrophs	<i>Vibrio oxalaticus</i>	formate
	<i>Pseudomonas oxalaticus</i>	
	<i>Paracoccus denitrificans</i>	methanol, methylamine, formate
	<i>Thiobacillus novellus</i>	
	<i>Microcycilus aquaticus</i>	methanol
	<i>Acromobacter</i> sp.	
	<i>Pseudomonas</i> sp.	
	<i>Mycobacterium</i> sp.	
	<i>Hydrogenomonas carboxy- dovorans</i>	carbon monoxide
	<i>Pseudomonas carboxydovorans</i>	
Phototrophs	<i>Seliberia carboxydohydrogena</i>	formate
	<i>Rhodopseudomonas palustris</i>	
	<i>Rhodopseudomonas gelatinosa</i>	
	<i>Rhodopseudomonas acidophila</i>	methanol
	<i>Rhodomicrobium vannielii</i>	
	<i>Rhodospirillum rubrum</i>	formate
	<i>Rhodospirillum tenue</i>	
Nonphotosynthetic, nonmethanogenic anaerobes	<i>Chloropseudomonas</i> sp.	carbon monoxide
	<i>Rhodopseudomonas</i> sp.	
	<i>Clostridium pasteurinum</i>	methane
	<i>Clostridium welchii</i>	
	<i>Clostridium thermoaceticum</i>	methane
	<i>Clostridium formicoaceticum</i>	
	Unidentified sulfate reducers	

App. Table 2.1
Classification of C₁ - Utilizing Microorganisms (continued...)

Methanogenic anarobes	<i>Methanobacterium</i> sp. <i>Methanospirillum</i> sp. <i>Methanococcus</i> sp. <i>Methanosarcina</i> sp.	methane, methanol, methyl- amines, formate dimethylsulfoxide, methane thiol
Miscellaneous bacteria	<i>Nocardia salmonicolor</i> <i>Bacillus</i> strain <i>Actinomyce</i> strain <i>Nocardia</i> strain <i>Bacillus pumilus</i> <i>Pseudomonas nonliquefaciens</i> <i>Bacterium thiocyanoxidans</i> <i>Thiobacillus thiocyanoxidans</i> <i>Pseudomonas stutzeri</i> <i>Thiobacillus thioparus</i> <i>E. coli</i>	carbon monoxide thiocyanate, cyanide thiocyanate dimethyl sulfoxide
Wide range of bacteria but especially ureolytic bacilli	<i>Bacillus pasteurii</i> <i>Bacillus sphaericus</i>	urea N-monomehtyl urea
Fungi	<i>Trichoderma lignorum</i> <i>Gliocladium deliquescens</i> <i>Paecilomyces varioti</i> <i>Aspergillus niger</i>	methanol, methylamine, formate methanol, formaldehyde dimethyl sulfoxide
Yeasts	<i>Kloeckera</i> sp. <i>Torulopsis glabrata</i> <i>Candida methanolica</i> <i>Torulopsis methanolovescent</i> <i>Candida boidinii</i> <i>Hansenula</i> sp <i>Pichia pinus</i> <i>Pichia methanolica</i> <i>Pichia pastoris</i> Pink yeast strains Many yeast species <i>Saccharomyces cerevisiae</i>	methanol methane methylamine dimethyl sulfoxide

Methanotrophs:

- Are primarily gram negative obligate aerobes
- Can grow only on methane
- May utilize methanol and dimethylether as a substrate
- Occur wherever methane is exposed to aerobic environments such as soil, sediments, natural waters, etc.
- May reach concentrations of 5E7/ml in sediments

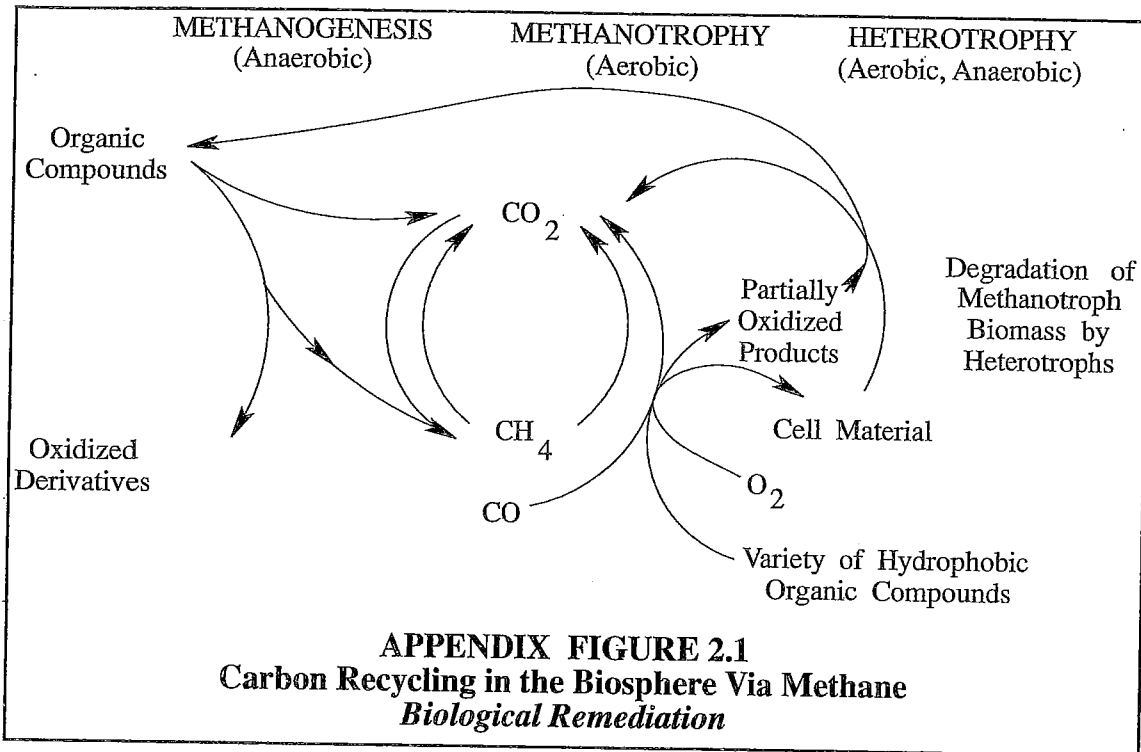
The roles of methanogens, methylotrophs, and methanotrophs are very significant in the environment because they not only convert the C₁ compounds to non toxic products, but also, in some cases, initialize the biodegradation of other organic compounds.

Methanotrophs have been found to:

- Degrade complex hydrophobic compounds which are not growth substrates
- Fix atmospheric nitrogen and oxidize ammonia to nitrite and nitrates

- Metabolize potent metabolic inhibitors like cyanide and carbon monoxide
- May remove pollutants, especially xenobiotic compounds

A schematic illustrating their importance in the environment is shown in Appendix Figure 2.1.



2.1.2. Multi-carbon metabolizers

Many microorganisms may not be able to use aliphatic hydrocarbons as growth substrates; however, they may oxidize them in the presence of other growth substrates. This phenomena, known as co-metabolism or co-oxidation, causes oxidation of many aliphatic hydrocarbons. *Pseudomonas mechanical*, for example, could oxidize ethane, propane, and butane in the presence of methane, but none of these gases could serve as a growth substrate for the organism.

Soil microbial communities growing on light aliphatic hydrocarbons have also been found to be capable of co-metabolically degrading trichloroethylene and related compounds normally recalcitrant to biodegradation in aerobic surface environments. This microbial process has high potential as a basis for methodologies for removal of chlorinated hydrocarbon solvents from polluted ground water, both *in situ* and in surface based systems.

A list of some of these microorganisms is provided in App. Table 2.2. Populations of these organisms vary with the habitat. For example, they may be more prevalent in oil polluted environments. Enrichment of hydrocarbonoclastic microbes in areas containing petroleum, has been applied in prospecting for oil and gas fields. Geographical location factors in determining population density of these microbes include availability of nutrients, type of climate, and other physical conditions.

App. Table 2.2
Genera of Bacteria and Yeasts the Reportedly Contain Aliphatic Hydrocarbon-Oxidizing Species

Bacteria	Yeasts
<i>Achromobacter</i>	<i>Candida</i>
<i>Acinetobacter</i>	<i>Cryptococcus</i>
<i>Actinomyces</i>	<i>Debaryomyces</i>
<i>Aeromonas</i>	<i>Endomyces</i>
<i>Alcaligenes</i>	<i>Hansenula</i>
<i>Arthrobacter</i>	<i>Mycotorula</i>
<i>Bacillus</i>	<i>Pichia</i>
<i>Beneckea</i>	<i>Rhodotorula</i>
<i>Brevibacterium</i>	<i>Saccharomyces</i>
<i>Corynebacterium</i>	<i>Selenotila</i>
<i>Flavobacterium</i>	<i>Sporidiobolous</i>
<i>Methylobacter</i> ^a	<i>Sporobolomyces</i>
<i>Methylbacterium</i> ^a	<i>Torulopsis</i>
<i>Methylococcus</i> ^a	<i>Trichnosporon</i>
<i>Methylocystis</i> ^a	
<i>Methylomonas</i> ^a	
<i>Methylosinus</i> ^a	
<i>Micromonospora</i>	
<i>Mycobacterium</i>	
<i>Nocardia</i>	
<i>Pseudomonas</i>	
<i>Spirillum</i>	
<i>Vibrio</i>	

^a Methane is the only hydrocarbon used as a sole carbon source. Aliphatic hydrocarbons larger than methane may be cooxidized.

2.2 Biodegradation Pathways for Typical Alkanes and Aromatics

Alkanes may occur as the source pollutant or be the product of prior multi-carbon substrates. Biodegradation pathways for the single carbon compounds are simpler and will aid in the understanding the pathways of the more complex compounds.

Methane is the most common C1 substrate involved in microbial metabolism since many compounds are converted into methane under anaerobic conditions.

The microorganisms involved include:

Methanogens - the class of microorganism that converts many organic compounds into methane under aerobic conditions

Methanotrophs - a class of microorganisms that converts methane into carbon dioxide in aerobic environments

Methylotrophs - more versatile nutritionally than Methanotrophs — cannot use methane as a carbon and energy source

Some of the other significant C1 compounds, which can also be utilized substrates, are listed in App. Table 2.3.

App. Table 2.3
C₁ Compounds of Major Environmental Importance

Compound	Formula	Comments
Methane	CH ₄	Ubiquitous end product of anaerobic fermentation processes. Generated by free-living and rumen-inhabiting methanogens.
Methanol	CH ₃ OH	Generated during the breakdown of natural methyl-esters and ethers (e.g., pectin) and released by methanotrophs.
Formaldehyde	HCHO	Common combustion product, intermediate microbial oxidation of other C ₁ compounds and methylated biochemicals (e.g., lignin)
Formate ion	HCOO ⁻	Present in plant and animal tissues. Common product of carbohydrate fermentation.
Formamide	HCONH ₂	Natural product formed from plant cyanides.
Carbon dioxide	CO ₂	Combustion, respiration, and fermentation end product. A major reservoir of carbon on Earth.
Carbon monoxide	CO	Combustion product, common pollutant. Product of plant, animal, and microbial respiration, highly toxic.
Cyanide ion	CN ⁻	Generated by plants, fungi, and bacteria. Industrial pollutant, highly toxic.
Dimethylether	CH ₃ OCH ₃	Generated from methane by methanotrophs. Industrial pollutant.
Methylamine	CH ₃ NH	Wide spread occurrence in tissues of plants, animals, fishes. Nitrogenous degradation products, excretion products.
Dimethylamine	(CH ₃) ₂ NH	
Trimethylamine	(CH ₃) ₃ N	
Trimethylamine-N- oxide	(CH ₃) ₃ NO	
Tetramethylammonium salts	(CH ₃) ₄ N ⁺	Halogen salts used as disinfectants.
Trimethylsulfonium salts	(CH ₃) ₃ S ⁺	Industrial chemical.
Urea	CO(NH ₂) ₂	Important animal excretion product.
Carbamic acid	NH ₂ COOH	Hydrolysis product of urea, fertilizer, N- methyl- and N, N-dimethyl derivatives used as pesticides.
N- Monomethylurea	CH ₃ NH.- CO.NH ₂	Nitrogenous degradation product.

Research has shown that the obligate methanotrophs convert methane to carbon dioxide by two electron oxidation steps, as shown in Figure 2.2

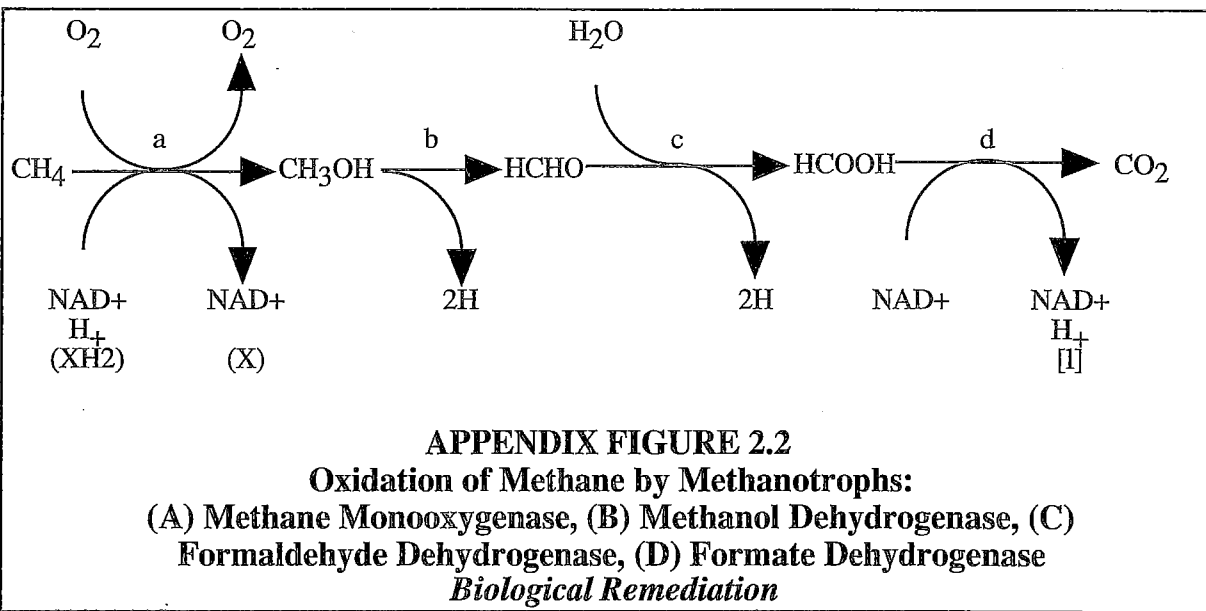
- Methane is converted to methanol in presence of oxygen.

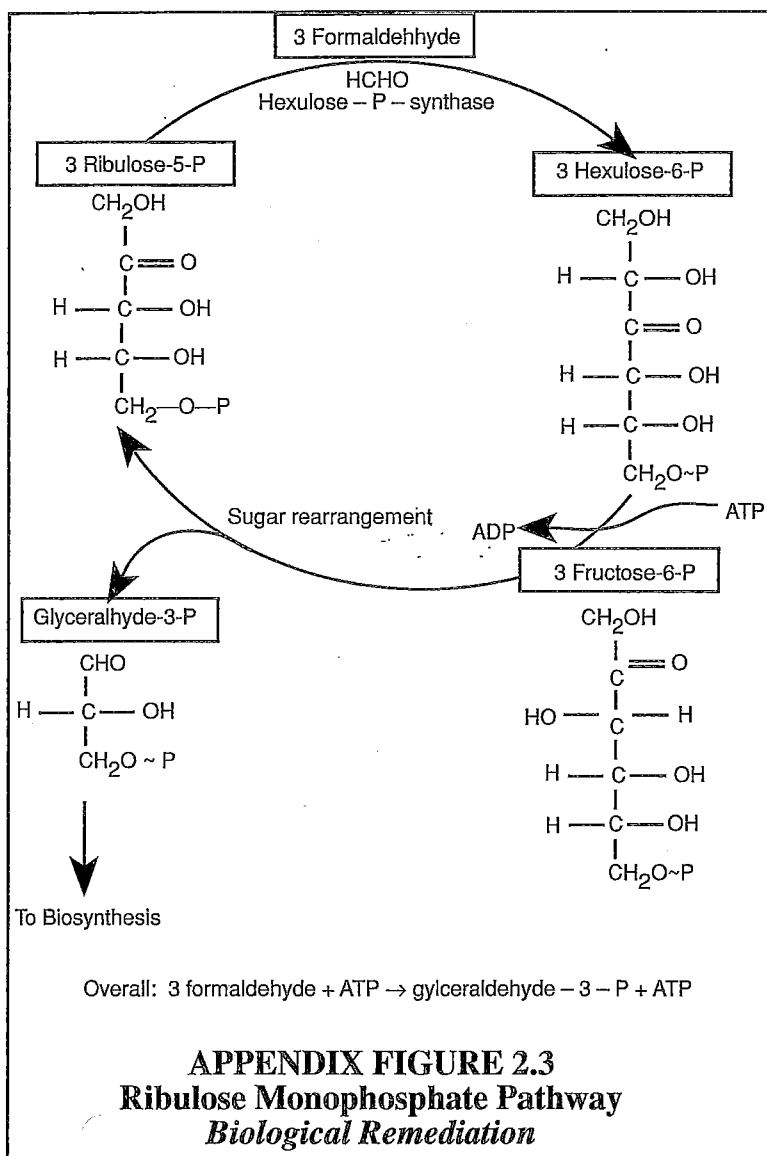
There is evidence to indicate that the oxygen in methanol is incorporated from molecular oxygen suggesting the involvement of an oxygenase. All methanotrophs have methane mono-oxygenase required for incorporating molecular oxygen in methane to yield methanol. The methane mono-oxygenases examined so far have extremely broad substrate specificities and can at least partially oxidize a wide variety of substances including carbon monoxide, ammonia, higher alkanes, alkenes and aromatic and alicyclic compounds. It can initiate biodegradation of many multi-carbon compounds which are otherwise non-growth substrates. In some cases oxidations are solely the result of mono-oxygenase activity as in the case of propane being converted to propene-oxide.

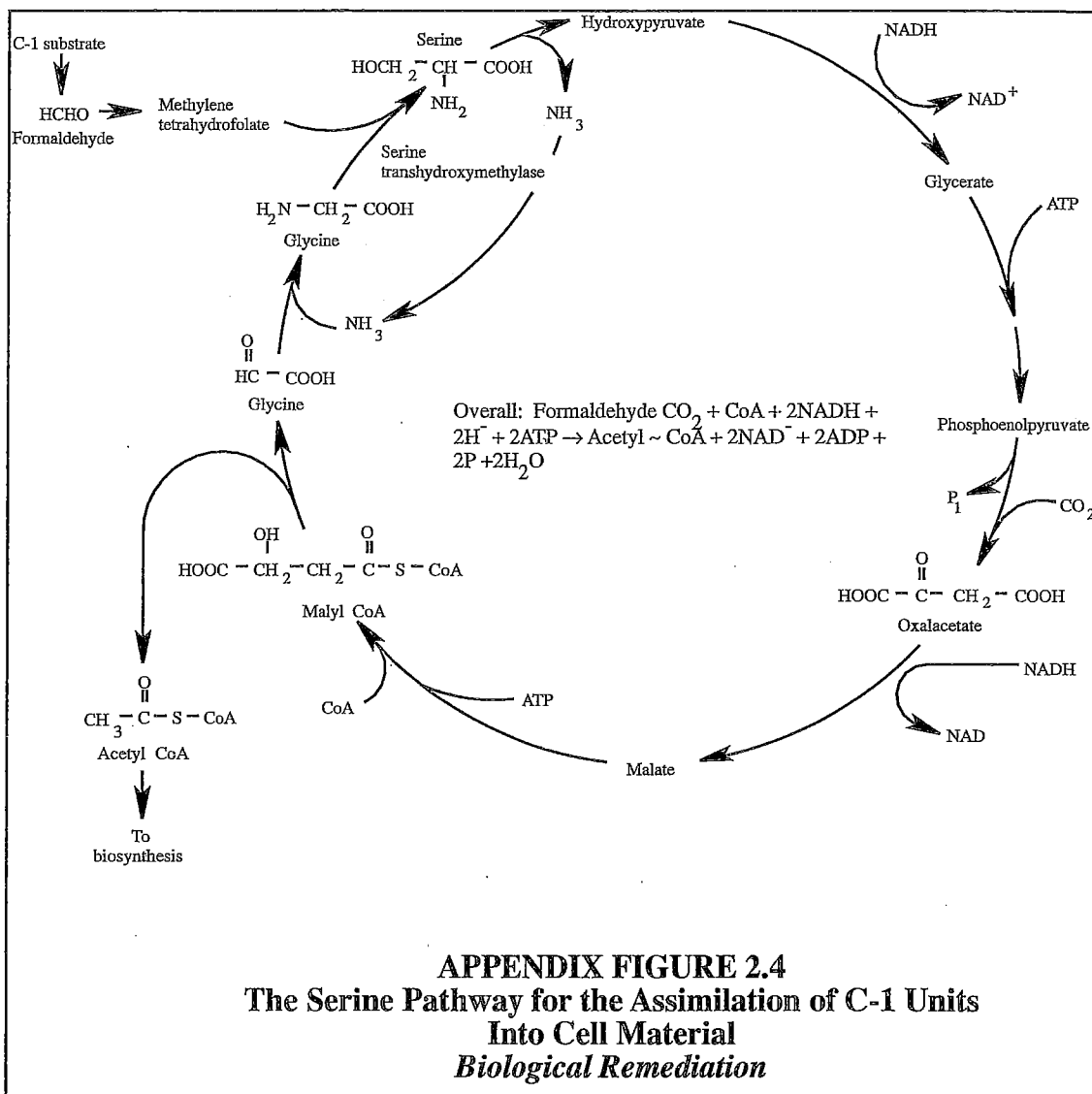
- Methanol is then subsequently converted to formaldehyde, formate, and, eventually, carbon dioxide by dehydrogenases

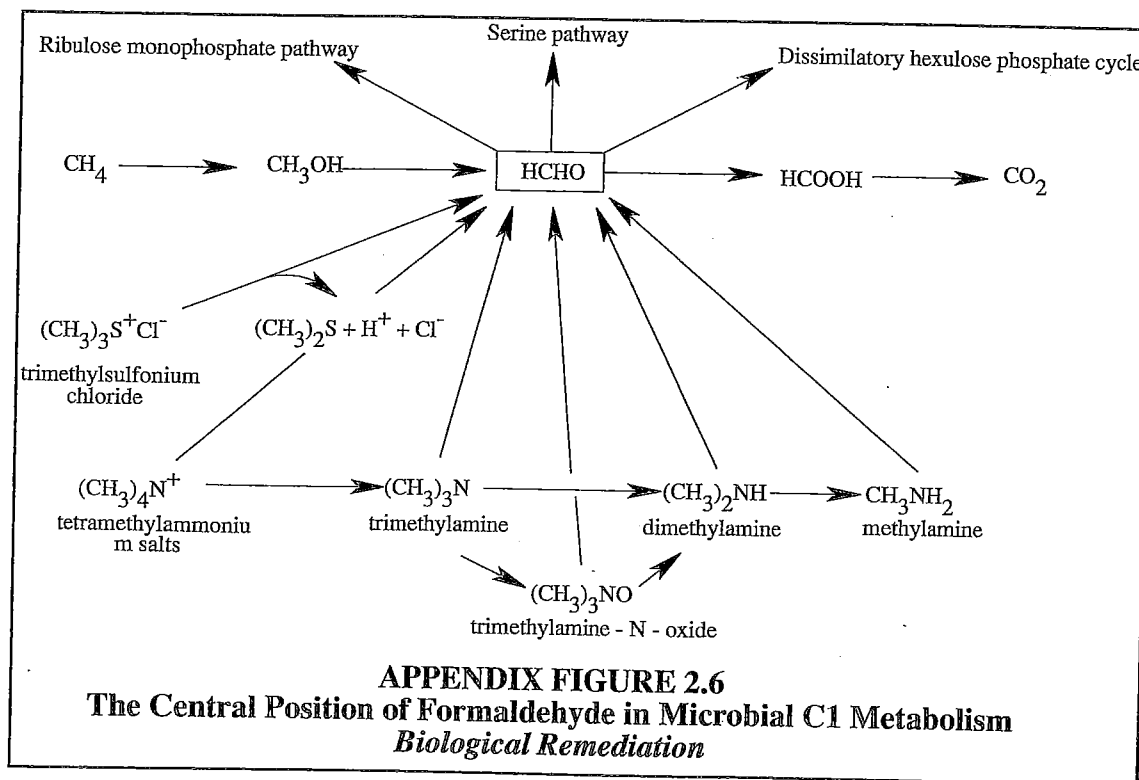
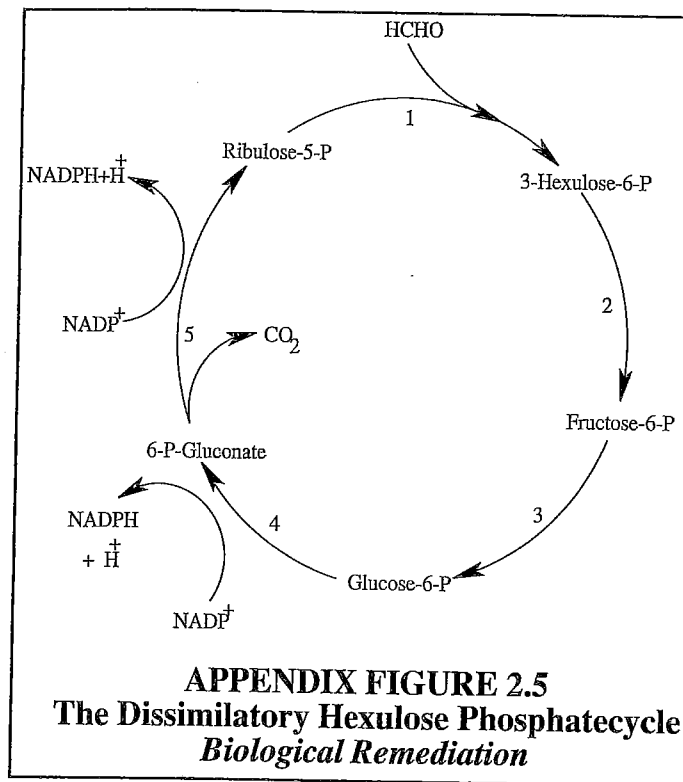
Formation of formaldehyde is of prime importance since many microorganisms incorporate carbon at the formaldehyde level into biomass via various pathways like ribulose monophosphate cycle (App. Figure 2.3), serine pathway (App. Figure 2.4), or dissimilatory hexulose phosphate pathway (App. Figure 2.5). Formaldehyde thus acts as the introductory point for the organic carbon into the biomass (App. Figure 2.6).

Many C1 compounds are converted to formaldehyde for incorporation into the biomass. Some of the formaldehyde formed is incorporated while the rest is converted to CO₂. In many species which use ribulose monophosphate pathway for formaldehyde fixation, a cyclic pathway for oxidation of formaldehyde, the dissimilatory hexulose phosphate pathway is operational.









In the case of aliphatic hydrocarbons, some generalizations can be made regarding their utilization for biodegradation:

- Aliphatic hydrocarbons assimilate more readily than aromatic hydrocarbons.
- Long chain hydrocarbons assimilate more readily than short chain hydrocarbons.

n-Alkanes, shorter than n-nonane, may be oxidized but are not assimilated usually.

For yeasts, there is an increase in the conversion of n-alkane to biomass and a decrease in the rate of oxidation with an increase in chain lengths after n-nonane. The reason for this behavior could be the toxicity of the short chain liquid hydrocarbons. This toxicity can be attributed to the greater solubility in the aqueous phase. The toxic levels in the aqueous phase could be reduced by partitioning the toxic hydrocarbon between aqueous phase and a second non-toxic hydrocarbon.

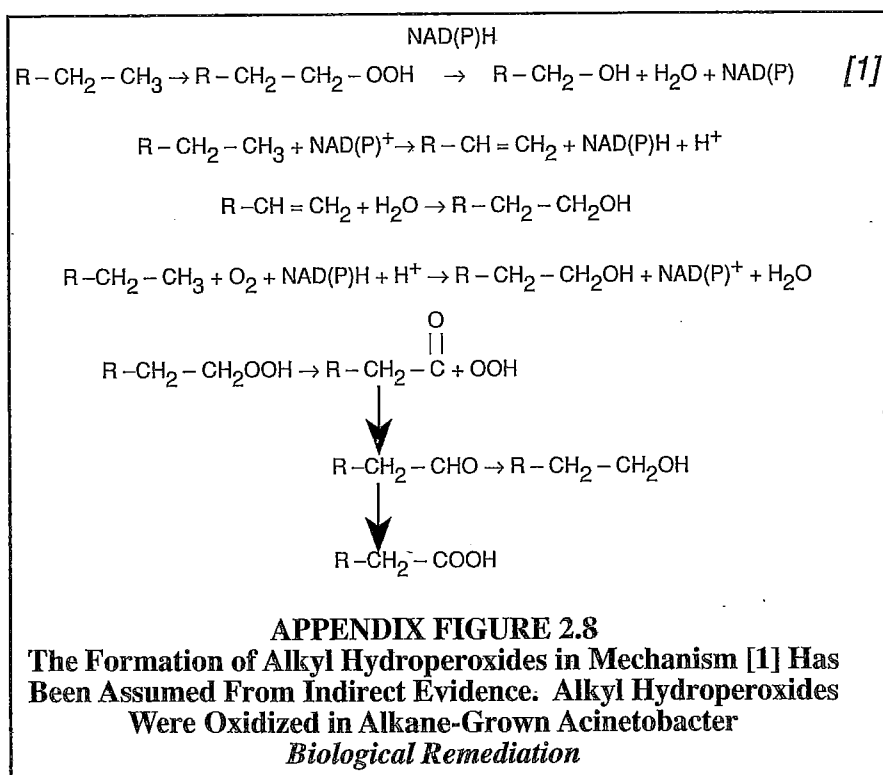
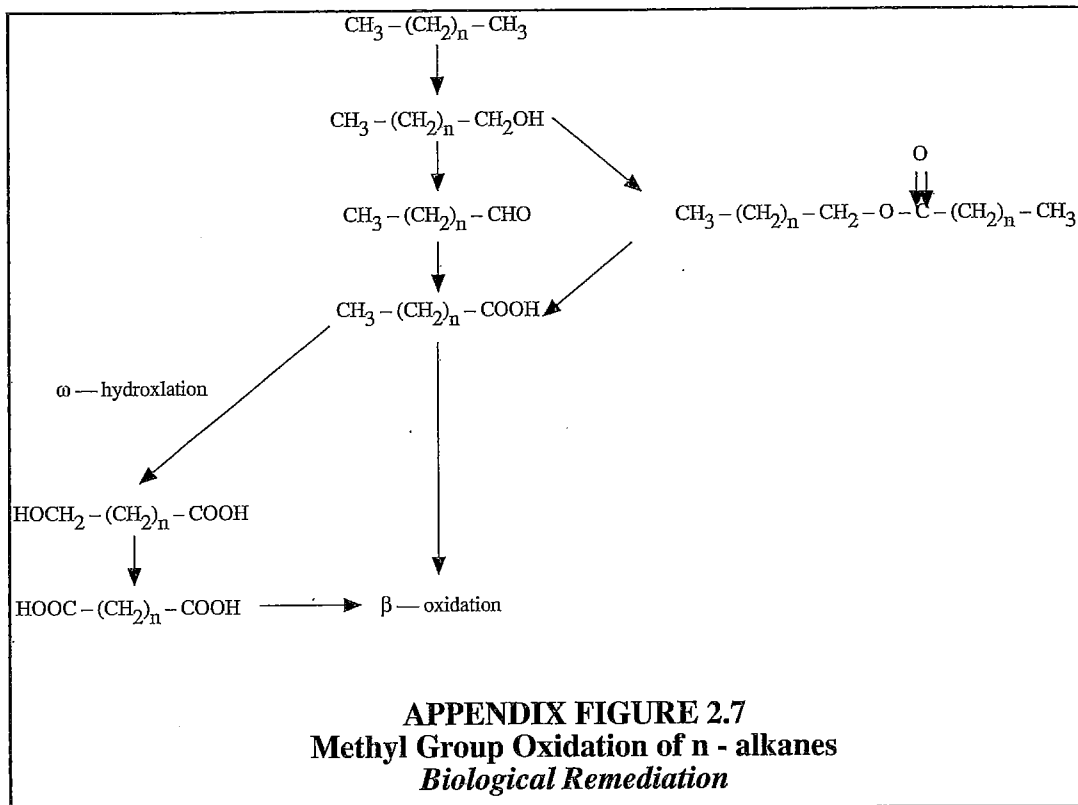
- Saturated aliphatic hydrocarbons degrade more easily than unsaturated ones
- Branched chain hydrocarbons degrade less readily than straight chain ones

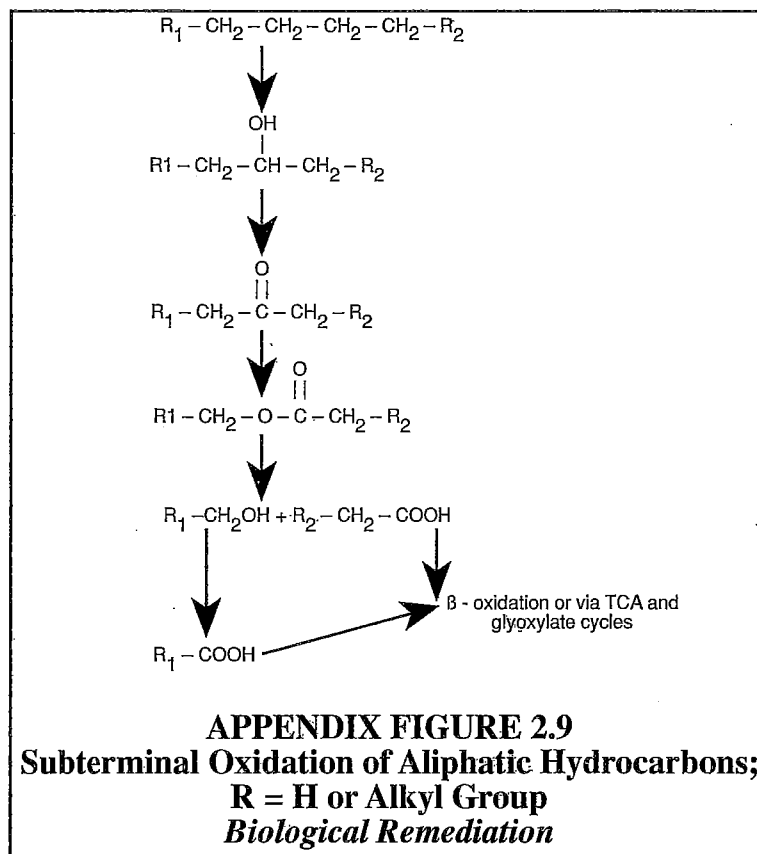
Of the many mechanisms proposed for the oxidation of aliphatic hydrocarbons, all propagate oxidation of the terminal methyl group. The original proposal of terminal oxidation was based on the detection of homologous fatty acids in the culture media. Presence of esters, presumably formed by alcohols and homologous fatty acids, confirms oxidation at C1 position. This also indicates that the terminal methyl group is first oxidized to a primary alcohol followed by subsequent oxidation to fatty acid. Once fatty acid is formed, it is subjected to β -oxidation yielding acetyl-CoA. Acetyl-CoA thus generated can be further oxidized via TCA cycle to yield energy or can be converted to hexose and other cell constituents via the glyoxylate cycle. Methyl group oxidation of n-alkanes is shown in Appendix Figure 2.7 as an example.

Some of the oxidative mechanisms are described by the mechanisms shown in Appendix Figure 2.8. There are problems associated with these mechanisms. For example, if the primary alcohols are formed by hydration of alkenes, as proposed by mechanism 2, then molecular oxygen should not be required for the oxidation. This definitely is not true since most of the hydrocarbon degradation processes have been known to utilize oxygen. If, on the other hand, primary alcohols are formed by way of oxygenation of alkenes then the oxidative products from n-alkanes and 1-alkenes should be the same, which, again, has been found to be incorrect.

The most widely accepted pathway for hydrocarbon degradation is mechanism 3. As indicated by this pathway, direct incorporation of oxygen is required for the formation of the primary alcohol. This requires presence of mixed-function oxidase or mono-oxygenase. Thus, organisms or systems having these enzymes can undergo aerobic oxidation easily.

Another mechanism, which is not so common or established is the sub-terminal oxidation of the methyl group. The phenomena was observed when propane and butane co-metabolized to acetone and 2-butanone, respectively. Subterminal oxidation is demonstrated by *Penicillium* species and strains of *Pseudomonas aeruginosa*. Existence of subterminal oxidation is also apparent when secondary alcohols and methyl-ketones are obtained from oxidation of n-alkanes by methane utilizing bacteria. A schematic showing the subterminal oxidation of n-alkane is shown in Appendix Figure 2.9. In this mechanism, studies conducted on 2-tridecanone oxygenase demonstrated that the oxygen atom inserted during conversion of the ketone to an ester is on the side of carbonyl group with the longest alkyl group.





3. ANAEROBIC BIODEGRADATION

3.1 Types of Microorganism

Anaerobic environments, or environments of low reduction potential, include:

- Muds and other sediments of lakes
- Rivers and oceans
- Bogs and marshes
- Water logged sites
- Deep underground areas (such as oil pockets)
- Most ground water environments

Low reduction potential - is due to a lack of replacement of oxygen, necessary for the bacteria that use oxygen for respiration, and will cause the habitat to become anaerobic.

Anaerobiosis occurs in only two groups of microorganisms:

- Protozoa
- Bacteria

Clostridia - the best known obligately anaerobic bacteria, are a group of gram positive spore forming rods which are wide spread in soils and lake sediments.

Other obligately anaerobic bacteria are found among the methanogenic bacteria and the sulfate reducing bacteria.

Some substrates metabolized/oxidized by methylotrophic bacteria and their important characteristics are given in App. Table 3.1 and App. Table 3.2

Characteristics of sulfate and sulfur-reducing bacteria are provided in App. Table 3.3.

Sensitivity to oxygen greatly varies among obligate anaerobes. Some organisms can tolerate traces of oxygen while others cannot.

App. Table 3.1
Substrates Used by Methylotrophic Bacteria*

Substrates used for growth	Substrates oxidized, but not used for growth (co-metabolism)
Methane, CH ₄	Ammonium, NH ₄ ⁺
Methanol, CH ₃ OH	Ethylene, H ₂ C=CH ₂
Methylamine, CH ₃ NH ₂	Chloromethane, CH ₃ Cl
Dimethylamine, (CH ₃) ₂ NH	Bromomethane, CH ₃ Br
Trimethylamine, ((CH ₃) ₃ N	Higher hydrocarbons (ethane, propane)
Tetramethylammonium, (CH ₃) ₄ N ⁺	
Trimethylamine N-oxide, (CH ₃) ₃ NO	
Trimethylsulfonium, (CH ₃) ₃ S ⁺	
Formate, HCOO ⁻	
Formamide, HCONH ₂	
Carbon monoxide, CO	
Dimethyl ether, (CH ₃) ₂ O	
Dimethyl carbonate, CH ₃ OCOOCH ₃	

*A single isolate does not use all of the above, but at least one methylotrophic bacterium has been reported to oxidize each of the listed compounds.

App. Table 3.2
Some Characteristics of Methane-Oxidizing Bacteria

Organism	Morphology	Flagellation	Resting Stage	Internal Membranes*	Carbon Assimilation Pathway	DNA (Mole % GC)
Methylomonas	Rod	Polar	Cyst-like body	I	Ribulose monophosphate	50-54
Methylobacter	Rod	Polar	Thick-walled cyst	I	Ribulose monophosphate	50-54
Methylococcus	Coccus	None	Cystlike body	I	Ribulose monophosphate	62
Methylosinus	Rod or vibrioid	Polar tuft	Exospore	I	Serine	63-66
Methlocystis	Vibrioid	None	Poly- β -hydroxy-butyrate-rich cyst	I	Serine	—
Methylobacterium	Rod	Nonmotile	None	I	Serine	58-66

* Internal membranes: Type I, bundles of disk-shaped vesicles distributed throughout the organism; Type II, paired membranes running along the periphery of the cell.

App. Table 3.3
Characteristics of Sulfate- and Sulfur-Reducing Bacteria

Genus	Characteristics	DNA (mole % GC)
Group I sulfate reducers: Non-acetate oxidizers		
<i>Desulfovibrio</i>	Polarly flagellated, curved rods, no spores; Gram negative; contain desulfovibrin; seven species recognized; one thermophilic	46-61
<i>Desulfomonas</i>	Long, fat rods; nonmotile; no spores; Gram negative; contain desulfovibrin; habitat, intestinal tract; one species	66-67
<i>Desulforomaculum</i>	Straight or curved rods; motile by peritrichous or polar flagellation; Gram negative; desulfovibrin absent; produce endospores; four species, one thermophilic, one species capable of utilizing acetate as energy source	37-46
<i>Archaeoglobus</i>	Achaebacterium; extreme thermophile, temperature optimum, 83°C; contains some unique coenzymes of methanogenic bacteria, makes small amount of methane during growth; H ₂ , formate glucose, lactate, and pyruvate serve as electron donors, SO ₄ ²⁻ , S ₂ O ₃ ²⁻ , or SO ₃ ²⁻ as electron acceptor; one species	46
<i>Desulfobulbus</i>	Ovoid or lemon-shaped cells; no spores; Gram negative; desulfovibrin absent; if motile, by single polar flagellum; utilizes only propionate as electron donor with acetate + CO ₂ as produces; one species	59

App. Table 3.3
Characteristics of Sulfate- and Sulfur-Reducing Bacteria (continued...)

Group II sulfate reducers: Acetate-oxidizers		
<i>Desulfobacter</i>	Rods; no spores, Gram negative; desulfoviridin absent; if motile, by single polar flagellum; utilizes only acetate as electron donor and oxidizes it to CO ₂ via the citric acid cycle; one species	45
<i>Desulfobacterium</i>	Rods, some with gas vesicles, marine; capable of autotrophic growth via the acetyl-CoA pathway, three species	—
<i>Desulfococcus</i>	Spherical cells; nonmotile; Gram negative; desulfoviridin present, no spores; utilizes C ₁ -C ₁₄ fatty acids as electron donor with complete oxidation to CO ₂ ; capable of autotrophic growth via the acetyl-CoA pathway; one species	57
<i>Desulfonema</i>	Large filamentous gliding bacteria; Gram positive; no spores; desulfoviridin present or absent; utilizes C ₂ -C ₁₂ fatty acids as electron donor with complete oxidation to CO ₂ ; capable of autotrophic growth via the acetyl-CoA pathway; one species	34-41
<i>Desulfosarcina</i>	Cells in packets (sarcina arrangement); Gram negative; no spores; desulfoviridin absent; utilizes C ₂ -C ₁₄ fatty acids as electron donor with complete oxidation to CO ₂ ; capable of autotrophic growth via the acetyl-CoA pathway; one species	—
Dissimilatory sulfur reducers:		
<i>Desulfuromonas</i>	Straight rods, single lateral flagellum; no spores; Gram negative; does not reduce sulfate; acetate, succinate, ethanol, propanol used as electron donor; obligate anaerobe; three species	50-63
<i>Campylobacter</i>	Curved, vibrio-shaped rods; polar flagella; Gram positive; no spores; unable to reduce sulfate but can reduce sulfur, sulfite, thiosulfate, nitrate, or fumarate anaerobically with acetate or a variety of other carbon/electron donor sources; facultative aerobe	40-42

3.2 Biodegradation Pathways for Typical Alkanes and Aromatics

Hydrocarbon degradation is usually an aerobic process. Evidence including observations on petroleum hydrocarbon, indicates that oxygen is required for the biodegradation. Yet, reports on anaerobic degradation and consideration of anaerobic pathways persist in literature. Anaerobic degradation of aromatics, for example, is well established.

Anaerobic degradation of aromatics can occur by:

- Photodegradation
- Nitrate respiration
- Methanogenic fermentation

Benzene and several of its alkyl derivatives, all of which have been considered to be recalcitrant to anaerobic biodegradation earlier, have been observed to undergo relatively rapid and extensive biodegradation in anoxic aquifer materials.

Presence of anaerobic degradation of aliphatic hydrocarbons however, is not so well established, though there is evidence of its existence.

A strain of *Pseudomonas*, for example, was found to grow on n-alkanes under anaerobic environment when nitrate was present. This strain could not grow aerobically with other conditions being the same. An NAD dependent n-alkane dehydrogenase was isolated from the extracts of this specie. In purified form this

enzyme could convert n-decane to 1-decene, which, as explained below, is the first step for anaerobic oxidation of alkanes.

In another study *Desulfovibrio*, a sulfate reducing obligate anaerobe, was found to degrade aliphatic hydrocarbon anaerobically. A study was made to assess the thermodynamic feasibility of utilizing hydrocarbons as energy source for sulfate reducers. It was found that none of the long chain hydrocarbons are adequate as a sole energy source for the microbes but can be cometabolized in the presence of other energy sources.

In a study on anaerobic oxidation of methane by sulfate reducers, it was found that the microbes could not oxidize methane to carbon dioxide unless acetate was present in the medium. It was also established that acetate, and not methane was assimilated by the microbes. It was concluded that in this case acetate was acting as the carbon source with methane as the electron donor (or source of energy) and sulfate as the terminal electron acceptor. It would appear from this study that if methane, which is considered biologically inert under anaerobic conditions, can be oxidized then other aliphatic hydrocarbons could be oxidized too. Studies on oxidation of hexadecane under similar conditions, however has contradicted this phenomena.

Anaerobic oxidation proceeds in a similar manner to aerobic oxidation.

- The alkane is first dehydrogenated to the corresponding alkene
- The alkene is then hydrated at the double bond to yield an alcohol

This step is not well documented and there is no evidence that the oxygen in the alcohol is derived from the water molecule. There is evidence however that the conversion from alkene to alcohol can be established in the absence of molecular oxygen by a hydrase mechanism.

- The next step in the anaerobic oxidation is oxidation of the alcohol to the corresponding fatty acid
- The β -oxidation yields acetyl-CoA
- A glyoxylate or TCA cycle, then, yields cell mass or energy respectively

The initial hydroxylation of alkanes and alkenes seems to be the rate limiting step. Once the alcohol is formed further oxidation is achieved easily as long as there is a balance between oxidation and reduction.

In a study done on cycling of natural and xenobiotic forms of carbon in anaerobic subsurface environment, biodegradation of halogenated aromatics was observed. Carbon and electron flow was primarily through methanogenesis and sulfate reduction. The anaerobic decomposition of halogenated aromatic substrates was preceded by the initial removal of aryl halides under methanogenic conditions. However, the anaerobic metabolism of alkylated aromatic compounds proceeded via methyl hydroxylation reactions.

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