January 1992

Bioengineering for Water Cleanup: State-of-the-Art Assessment

Judith L. Sims

Ronald C. Sims

Jon S. Ginn

Bioengineering For Water Cleanup:

State-of-the-Art Assessment

by

Judith L. Sims, Ronald C. Sims, and Jon S. Ginn

Utah Water Research Laboratory

Utah State University

Logan, Utah 84322-8200

November, 1992

National Geographic Society Grant No. 4787W-92

TABLE OF CONTENTS

LIST OF TABLES

BIOENGINEERING FOR WATER CLEANUP:

STATE-OF-THE-ART ASSESSMENT

1.0 INTRODUCTION

A national sense of urgency with regard to cleaning up contaminated water has been motivated by recent legislation promulgated as a result of adverse public health and environmental impacts. However, current technologies for cleaning up contaminated water containing hazardous organic substances are often expensive, inappropriate for specific conditions, or ineffective in handling complex mixtures of pollutants[203] Bioengineering (biological engineering), in the context of water cleanup, is the use of biological principles of thermodynamics, kinetics, and toxicology for applications to the conceptualization, design, management, monitoring, and economic evaluation of engineered systems to accomplish treatment of water in order to protect public health and the environment from the adverse effects of target chemicals. Non-pathogenic organisms, including bacteria, fungi, and algae, individually too small to be seen by the unaided human eye (referred to as microorganisms), are the primary agents utilized by the biological engineer to remove chemicals from contaminated water. Some of the most promising technologies for solving problems associated with contaminated water involve the use of these biological treatment systems, i.e., use of the technology referred to as bioremediation. The biodegradation of organic compounds has been used for hundreds of years for the treatment of human wastewaters (e.g., using sewage farms and trickling filters), with treatment effectiveness determined on the ability of the treatment process to reduce the levels of oxygen-demanding substances and nutrients in the waste effluent (in order to prevent putrefaction and eutrophication of the receiving waters) as well as to reduce the pathogenic nature of the waste effluent. In 1907, microorganisms were first identified as playing an important part in the biodegradation process. In 1914, the now commonly-used activated sludge process for the treatment of wastewater was developed in England. Since that time, collaboration by microbiologists, biochemists, environmental engineers, and chemical engineers has developed the field of bioremediation to its

present state as an effective means for treatment of domestic wastes and selected industrial waste streams. However, the application of existing bioengineering systems and processes, which have been developed for the removal of organic materials in general, to the treatment of specific xenobiotic, recalcitrant and persistent organic compounds found in contaminated water, is an area of high-priority research in the environmental field. Reactor studies have shown that the prediction of the fate of a single organic compound in a complex wastewater is difficult to achieve. Also, water with many types of contaminants is more difficult to remediate than a water contaminated with only one pollutant. Challenges still remain for the successful use of bioremediation in the treatment of specific organic pollutants within current regulatory requirements.

Bioremediation, as accomplished through biodegradation, refers to the remedial process by which an organic compound, called the parent compound (which includes both naturally occurring and xenobiotic compounds), is biotransformed (i.e., mineralized) in a treatment reactor (which may be an above-ground reactor (contained vessel) or an *in situ* treatment system) by the action of living microorganisms or their enzymes to carbon dioxide, water, and other inorganic constituents (if elements other than carbon, hydrogen, and oxygen are present in the parent compound), resulting in ultimate biodegradation or mineralization of the parent compound. [182, 183] A portion of the constituents of the parent compound will also be assimilated into the biomass of the organisms in a process called cell synthesis, or anabolism. If the microorganism produces energy during the degradative process, the process is referred to as catabolism. Though bioremediation is usually accomplished under aerobic conditions, anaerobic metabolic activities are used in some bioremedial techniques [184, 196-198]; anaerobic degradation results in the transformation of parent compounds to intermediates that are more easily biodegraded upon exposure to an environment containing molecular oxygen (O_2), but that will accumulate as incompletely oxidized organic substances such as organic acids and gaseous products such as

methane or hydrogen gas in an environment without molecular oxygen.

Under natural conditions, biodegradation may not proceed to ultimate biodegradation. Since biodegradation is frequently a stepwise process involving many enzymes and many species of microorganisms, the parent compound may be only changed to an intermediate transformation product. This transformation product may be more, less or equally as toxic as the parent compound, as well as more or less mobile in the environment, thus generating its own environmental and health consequences. [196] Acceptable biodegradation occurs when the parent compound is converted to the extent that undesirable properties are no longer manifested [12]. Information on detoxification of a parent compound is obtained using chemical and bioassay analyses.[186, 128] Before bioremediation is implemented at a contaminated site, degradation pathways for specific constituents present should be identified, and/or detoxification demonstrations using bioassays should be conducted to ensure that environmental and health protection can be achieved. During performance of a bioremedial process, monitoring should be implemented to ensure that toxic biotransformation products are not accumulating in the system or in the effluents.

Complete degradation of a specific organic compound usually requires an association, or consortia, of microorganisms, in which individual types of organisms carry out different specialized reactions that, when combined, can lead to the complete mineralization of a specific compound.[196] Biological systems are complex mixtures of thousands of biochemical reactions being conducted by many biological organisms; this complexity produces an outstanding ability for the systems to adapt to the treatment of a wide variety of pollutants, with the microorganisms using the pollutants as energy sources for metabolic and reproductive activities. [158] The release of large quantities of synthetic compounds into the environment has resulted in the evolution of new degradative functions by indigenous microorganisms [40], which may have resulted from the transfer of genetic materials, since microbial populations in nature seem to be capable of

substantial movement of genes between both the same and different genera and species.[96, 188] Interacting microbial consortia may also have evolved to degrade compounds introduced to the environment, since a community of microorganisms is more likely to result in the degradation of a specific waste constituent than a single microorganism.[188] New techniques developed in the field of genetic engineering are enabling the bioengineer to develop microorganisms with new degradative capabilities, rather than depend on natural adaptations by indigenous microorganisms.

Where possible, bioremedial technologies are developed to utilize indigenous microorganisms that have been demonstrated to metabolize pollutants present in a specific contaminated water or bioreactor system. In these cases, the number and/or rate of degradative activity of the microorganisms, and thus the speed at which a pollutant is broken down, may be increased in several ways, such as by adding nutrients or other amendments to the contaminated water or bioreactor system, in a process referred to as biostimulation. In other cases, acclimated or genetically engineered microorganisms known to metabolize the specific pollutants present can be introduced, if necessary, to stimulate biodegradation, in a process referred as bioaugmentation.

Bioremediation is an attractive remedial technique because it is a "natural process," and the residues from biological processes, including the degradation of xenobiotic compounds, are usually geochemically cycled through the environment as harmless products (e.g., carbon dioxide in the carbon cycle). The use of bioremediation, especially when used for *in situ* ground-water cleanup, minimizes site disruption and reduces or eliminates costs associated with transportation, handling, and disposal of recovered contaminants. In addition, compared to other physical or chemical processes used to treat hazardous wastes, in which contaminants are merely transferred from one environmental medium to another, bioremediation can degrade and destroy the target chemicals.

Common assumptions concerning bioremediation often include unrealistic expectations of

what the technology can accomplish. Though microorganisms have been demonstrated in the laboratory and at some contaminated sites to be extremely versatile in destroying organic compounds that are major environmental pollutants, non-specialists often assume that nearly all wastes and sites contaminated with biodegradable contaminants can be bioremediated. [3, 172] They may believe that if microorganisms with appropriate metabolic capabilities are added to any contaminated environment, if growth of indigenous organisms is enhanced with nutrients or oxygen, or if a supplemental carbon source is added to encourage cometabolism of contaminants, the site or waste can be successfully treated with bioremediation. However, many compounds that are readily destroyed by microorganisms in the laboratory may not be so easily degraded in a contaminated environment, i.e., the determination of whether or not a chemical is biodegradable reflects laboratory knowledge more often than it reflects engineering information and field experience (CA). For example, microorganisms that can degrade compounds that are considered recalcitrant may exist in natural environments, but they may not be present at a specific site where the recalcitrant compounds occur. Other substances may be present that are toxic to the microorganisms, or the environment may contain or be subject to biologically unfavorable conditions that hinder or prevent bioremediation. Microbial activity is often impeded by either very high or very low concentrations of the target chemical. [192] In addition, the target chemicals may be sorbed, dissolved in nonaqueous phase liquids (NAPLs), present in a physically inaccessible state, or bound in some way that prevents microorganisms with biodegradative enzymes from accomplishing biodegradation of the chemicals by preventing transport of the chemicals into the bacterial cell [3]. Some pollutants are resistant to biological degradation due to their size or chemical composition.[192] Therefore, physical and chemical characteristics, as well as the biological characteristics, of an engineered bioremedial system will determine the rate and extent of biological remediation by controlling the expression of inherent microorganism capabilities of the system. The success of bioremediation is site-specific, and a thorough understanding of the

biological, physical, and chemical characteristics of the specific system to be remediated is required. Ultimate limitations to the use of bioremediation for a specific contaminated water source will usually be related to: (1) time required for cleanup, (2) level of cleanup attainable, and (3) cost of cleanup using bioremediation.

Bioremediation in many cases should have an economic advantage for cleanup of contaminated water because of low capital, energy, and materials costs. [192] Bioremediation is also relatively "low-tech", does not have intensive labor requirements, and does not require costs for transportation of the hazardous contaminants. However, sometimes this cost advantage may be offset by factors such as high testing costs to characterize the system and possible limiting factors, additional technologies required for use in a treatment train to address multiple contaminants, long periods of time required to accomplish cleanup to regulatory levels, monitoring costs to provide the ability to quickly discover problems and implement changes, and contingency costs to cover possible upsets and failures in the system that may result in the system to stop performing according to specifications. [192] However, since estimated costs to cleanup present contaminated sites is so large, the use of bioremediation as a potentially inexpensive and easy technique is being recommended for development by the U.S. Environmental Protection Agency as a means to save billions of dollars in remedial costs. [71, 192]

Though bioremediation is a potentially efficient and cost effective remedial technology, there are still many research questions remaining before this technology can reach its full potential for use. In this state-of-the art assessment of bioengineering the cleanup of contaminated waters, the following areas are addressed: (1) important technical issues; (2) bioengineering technologies; (3) important regulatory issues; and (4) current state of knowledge regarding applications and limitations for bioengineering, with recommendations for future approaches for water cleanup. This review addresses the bioremediation of waters that have been contaminated in the past, such as polluted ground water, as well as bioremediation of wastewaters, which is used

at the source to prevent contamination of ground and surface waters. This review does not address the use of microorganisms for the conversion of toxic forms of metals to less toxic forms, nor does it address the use of microorganisms for the accumulation of toxic metals, resulting in their removal from a contaminated water system, though microorganisms do have the capabilities to accomplish these potentially useful remedial actions. [192]

2.0 TECHNICAL ISSUES

2.1 Introduction

In real world situations, the extent of biodegradation within a fixed time limit is an important component in the determination of the feasibility of use of bioremediation for a specific waste, i.e., biodegradation should be considered and evaluated as a rate process. For example, biodegradation of some compounds, such as urea to ammonia and CO_2, can be accomplished in seconds or minutes, biodegradation of lignin by white-rot fungi may take months, while tree resins may resist biodegradation for centuries. Basic engineering questions related to the time required for treatment and the final effluent concentrations that can be achieved cannot be reliably answered for biological treatment of many environmentally significant chemical compounds, even when prior laboratory or pilot-scale studies have been implemented. [20]

An example of the lack of integration of science and engineering disciplines regarding biological treatment concerns the use of pure-culture microorganism studies (one organism type) by scientists versus the use of mixed-culture systems from natural environments by engineers.[20] Pure-culture studies, often with only one carbon source, have been used to characterize biological degradation of many compounds (thousands of papers have been published in this area; reviews of these studies are available. [39, 80, 108-110, 125, 169] However, the mixed-culture microbial systems used in waste treatment systems are much more complex, and their system behavior and performance is much more difficult to predict. The design of biotreatment systems by the bioengineer has traditionally focused on the use of mixed-culture systems for the removal of

organic materials in general, as measured as the removal of biochemical oxygen demand (BOD), chemical oxygen demand (COD), and/or total organic carbon (TOC). However, the kinetics of removal of specific organic compounds may be very different from and unrelated to the removal of these overall parameters. Therefore the focus of environmental microbiology, which forms the microbial basis of biological engineering technologies, has traditionally been supportive but insufficient for solving the types of problems inherent in complex environmental systems.

Bioengineering approaches have often been based upon inappropriate hypotheses. For example, the most commonly used test for biodegradability that is used in bioremediation systems has been and still is the disappearance test, i.e., if the target chemical was not found or its amount decreased in the water, the observation was incorrectly interpreted as biodegradation. This functional definition of bioremediation has given a false sense of security, since the contaminant may not be completely transformed to harmless by-products. For example, the commonly-used cleaning solvents, tri- and tetrachloroethylene, may be transformed into the leukemia-causing agent, vinyl chloride, a persistent intermediate in anaerobic (oxygen-deficient) environments.

Bioengineering methods have also often been based upon inadequate methods of measurement of biodegradation. Using the measurement of disappearance from water as the criterion for bioremediation also ignores the roles of competing mechanisms that influence the fate of a target compound, such as stripping/volatilization, sorption, and non-biological (abiotic) chemical reactions. In the above example, both trichloroethene and vinyl chloride have strong tendencies to volatilize. Therefore, measurement of physical abiotic loss mechanisms and partitioning of target organic chemicals into other environmental compartments in a contaminated water source or treatment system should be used in conjunction with conventional degradation studies to ensure that information generated from the investigation of degradation represents only biological degradation of target compounds, and not other possible disappearance mechanisms of the chemicals in the system.

Thus, the prediction of the effectiveness of a bioremedial process for a specific organic

compound at field-scale is unfortunately, in many ways, still more of an art than a science. J.W. Blackburn, in a review of the uncertainties associated with the prediction of performance of mixed-culture systems at the reactor level, ecological level, cellular level, and molecular level [20], has proposed the following research approach to reduce these uncertainties: (1) development of molecular tools for community structure analysis (e.g., by using gene probes), in order to determine more accurate kinetic relationships and greater scale-up reliability; and (2) development of improved lab-scale experimental protocols that will allow the identification of critical causative processes and mechanisms, with emphasis on potential adaptability of microbial systems, potential non-linearity of processes, experimental reactor design for system data collection, and instrumentation required for continuous data collection of important variables. Dr. Blackburn emphasized especially the difficulties of studying a complex, undisturbed operating system without disturbing the system.

2.2 Approaches to the Determination of Bioremediation Potential

Measurement of physical abiotic loss mechanisms and partitioning of organic constituents in a contaminated water source or treatment system should be used in conjunction with conventional degradation studies (i.e., the use of a chemical mass balance approach) to ensure that information generated from the investigation of degradation represents only biological degradation of parent compounds, and not other possible disappearance mechanisms of the constituents in the system.[142,185] Contaminated water is a complex system and may consist of several components, including: (1) the aqueous phase; (2) an interface with the atmosphere; (3) inorganic solids (e.g., sediments, suspended solids); (4) organic solids (e.g., microbial mass, organic humic materials); and (5) non-aqueous phase liquids (e.g., oils, grease). An organic waste constituent in a contaminated water source may be associated with one or more of these components. The environmental fate of the constituent is dependent on the phase with which it is associated. A chemical mass balance approach to the evaluation of the fate and transport of a

target chemical in a specific water system identifies mechanisms by which a chemical may be removed from the water compartment without being biodegraded, and therefore provides a tool for the biological engineer to develop more accurate information concerning the rate and extent of bioremediation, and avoids an incorrect interpretation of data based upon inadequate methods of measurement. Biotransformation results for specific compounds from laboratory studies are often very different from results observed in pilot-scale, full-scale, and even other laboratory studies when a chemical mass balance is not used because of the differences in relative volumes of water, sediment, oil, and air phases in different test systems.

Treatability studies for water sources contaminated with organic wastes are used to provide specific information concerning the potential rate and extent of bioremediation by providing information on fate and behavior of specific organic constituents among the phase components present at a specific contaminated site. [143] Treatability studies can be conducted in laboratory microcosms, in bench scale or pilot scale facilities, or in the field. To determine whether a specific contaminated water can be treated using bioremediation, information from treatability studies is combined with information concerning site and waste characteristics in order to determine potential applications and limitations of the technology.

Information from treatability studies is also used to prepare an approach to the engineering design and implementation of a bioremediation system for a specific contaminated water source. An engineering design to accomplish bioremediation is generally based upon information from simulations (e.g., mathematical modeling) or estimates of degradation reactions (both biotic and abiotic) and pathways of migration of chemicals. These simulations or estimates are generated from treatability data and site/water characterization data, in order to: (1) develop techniques to maximize mass transfer of chemicals affecting microorganism activity (addition of mineral nutrients, oxygen, additional energy sources, pH control products, etc., and removal of toxic products) in order to enhance bioremediation; and (2) design a cost-effective and efficient monitoring program to evaluate effectiveness of treatment.

2.3 Determination of Biodegradation Potential

During the performance of a treatability study, biodegradation, detoxification, and partitioning (immobilization) processes should be evaluated as they affect the fate and behavior of organic constituents in the specific contaminated water system. To assess the potential for biological degradation at a specific contaminated site, the treatability studies should incorporate both chemical mass balance and mineralization approaches to determine the environmental fate and behavior of the constituents in the specific water system. Rate of degradation is calculated by measuring the loss of the parent compound (chemical mass balance approach) and the production of carbon dioxide with time of treatment (mineralization approach), as well as production and disappearance of intermediate products (chemical mass balance approach). Abiotic (poisoned) controls are also used in order to evaluate the mechanism(s) of degradation. Results can be reported, for example, as rate/extent of biological degradation corrected for volatilization and for other abiotic losses. The degradation rate is often reported as half-life, which represents the time required for 50 percent of the compound to disappear, based upon a first-order kinetic model.

2.4 Assessment of Transformation/Detoxification

Transformation refers to the partial alteration of hazardous constituents into intermediate products. Intermediate products may be less toxic or more toxic than the parent compound, and therefore the rate and extent of detoxification of the contaminated water source should be evaluated.[186, 199] For example, the degradation pathway of the single chlorinated compound, trichloroethylene (TCE) leads to the production of six chlorinated volatile hydrocarbons. The degradation of tetrachloroethylene (PCE) leads to the production of seven chlorinated volatile hydrocarbons, while the degradation of 1,1,1-trichloroethane (1,1,1-TCA) leads to the production of four chlorinated hydrocarbons. Two of the metabolic products formed, vinyl chloride and 1,1,-dichloroethane (1,1-DCA), have been classified as a carcinogen and a probable carcinogen, respectively?[214] Vinyl chloride is the most persistent of the compounds under anaerobic

conditions, but can be rapidly degraded under aerobic conditions. [66, 94] Management of a bioremediation system to accomplish treatment of these compounds in order to protect human health and the environment should incorporate considerations of the detoxification of the parent compounds as well as their disappearance. For example, for these halogenated organic compounds, a bioremediation system could consist of maintenance of an anaerobic environment followed by aeration after anaerobic degradative processes have reduced the levels of parent compounds to acceptable levels.

To assess detoxification, bioassays may be used to quantify toxicity by measuring the effect of a chemical on a test species under specified test conditions. [128] The toxicity of a chemical is proportional to the severity of the chemical on the monitored response of the test organism(s). Toxicity assays utilize test species that include rats, fish, invertebrates, microorganisms, and seeds. The assays may utilize single or multiple species of test organisms. The use of a single bioassay procedure does not provide a comprehensive evaluation of the toxicity of a chemicals in a water/chemical-impacted system. Often a battery of bioassays is utilized that may include measurements of effects on general microbial activity (e.g., respiration, dehydrogenase activity, ATP analysis, CO_2 evolution) as well as assays relating to activity of subgroups of the microbial community (e.g., nitrification, nitrogen fixation, cellulose decomposition). Bioassays utilizing organisms from different ecological trophic levels may also be used to determine toxicological effects. However, use of a single assay as a screening test to identify relative toxicity reduction in the environment is a commonly used procedure. Assays using microorganisms are often used due to their speed, simplicity, ease in handling, cost effectiveness, and the ability to use a statistically significant number of test organisms, which is required to detect the effects of potentially toxic materials in the environment. [59, 127] .

Two microbial bioassays that have been used to evaluate toxicity of wastes in water systems are the Ames *Salmonella typhimurium* mammalian microsome assay and the Microtox™

test system. The Ames assay is a measure of the mutagenic potential of hazardous compounds [5, 134] and has been widely used to evaluate environmental samples. [186] A high correlation has been shown between carcinogenicity and mutagenicity, where about 90% of known carcinogens tested mutagenic in the Ames assay.[135] Special strains of *Salmonella typhimurium* that require histidine to grow are used to test for mutagenicity. When plated on a histidine-free medium, the only bacteria able to form colonies are those that have reverted to the "wild" state and are able to produce their own histidine. Without the addition of test chemicals, this back mutation occurs at a rate specific to each strain type (spontaneous reversion rate). The addition of chemicals that are mutagenic increases the reversion rate. Several dose levels of a chemical, mixture of chemicals, or an environmental sample are added to obtain a dose response. Some mutagens act directly on the bacterial cells while others require activation by mammalian microsomes. These microsomes are generally obtained from liver extracts of Aroclor 1254-induced rats (i.e., rats injected with the polychlorinated biphenyl (PCB), Aroclor 1254). The extract, referred to as the S-9 fraction, contains enzymes that metabolically convert certain chemicals to active mutagens, simulating the activity that occurs in living mammalian systems. Several strains of *Salmonella typhimurium* have been developed in order to detect different types of mutagens. The recommended strains for general mutagenicity testing include TA 97, TA 98, TA 100, TA 102. TA 97 and TA 98 detect frameshift mutagens. TA 100 detects mutagens causing base-pair substitutions, while TA 102 detects a variety of mutagens not detected by the other strains.

The Microtox™ assay is an aqueous general toxicity assay that measures the reduction in light output produced by a suspension of marine luminescent bacteria in response to an environmental sample.[32] Bioluminescence of the test organism depends on a complex chain of biochemical reactions. Chemical inhibition of any of the biochemical reactions causes a reduction in bacterial luminescence. Therefore, the Microtox™ test considers the physiological effect of a

toxicant and not just mortality. Results from the Microtox test have in many cases shown good correlation with other bioassays such as fish lethality tests and daphnid static bioassays. [31]

2.5 Assessment of Partitioning

Calculation of the rate of decrease of parent compound by itself does not provide complete information concerning mechanisms and pathways by which organic constituents are interacting within the water environment.[185] Further information is necessary to understand whether a constituent is simply transferred from one phase (e.g., solid phase) to another (e.g., air phase) through a process of interphase transfer, or is chemically altered so that the properties of the parent compound are destroyed. Evaluation of the fate of a constituent in a contaminated water source therefore also requires identification and measurement of the distribution of the constituent among the physical phases or components that comprise the system as well as differentiation of the mechanisms by which the constituent may be chemically altered in the system. The distribution among phases may be predicted with partitioning coefficients that describe the tendency of the waste constituent to be associated with, and to transfer among, particular environmental phases. Partition coefficients are calculated as the ratio of the concentration of a chemical in the solid, oil, or air phase to the concentration of a chemical in the water phase, and are expressed as K_o (oil/water, or K_{ow}, the octanol/water partition coefficient, which indicates the tendency to be associated with organic matter), K_h (air/water), and K_d (solid/water).

2.6 Use of Treatability Studies

Either laboratory microcosm, bench scale or pilot scale reactors, or full-scale reactors may be used to generate treatability data. The set of experimental conditions, e.g., temperature, waste concentration, etc., under which the studies were conducted should be presented along with experimental results.

Treatability studies usually represent optimum conditions with respect to mixing, contact of microorganisms with waste constituents, and homogeneous conditions throughout the treatability

reactor. Therefore, treatability studies provide information concerning potential levels of treatment achievable at a specific site. However, under full-scale operating conditions, the rate and extent of bioremediation is generally limited by such factors as rate of mass transfer of oxygen, nutrients, and other amendments to the contaminated soil, accessibility and bioavailability of the contaminants to the microbial population, removal of microbial degradation products, and environmental conditions. These limitations may not have been adequately addressed in treatability testing, due to difficulties in simulating field-scale conditions.

2.7 Control and Optimization of Bioremediation

The primary means a bioengineer has for controlling and optimizing biodegradation processes in treatment systems are by selecting appropriate microorganisms and by providing proper environmental conditions, i.e., using bioaugmentation and/or biostimulation as appropriate for the specific waste and system. Examples of the use of bioaugmentation and biostimulation are given in Tables 1 and 2. A new tool for use in bioremediation is genetic engineering, which may potentially produce microorganisms that are more robust than natural strains and thus result in improvements in process improvement. However, at this time, if a bioengineer is to improve and optimize performance of a bioremedial system, he/she must develop a complete understanding of the biology, chemistry, and engineering involved in bioremedial processes.

The determination of effectiveness of biostimulation or bioaugmentation by a certain amendment or environmental change is not necessarily a straight-forward procedure. For example, a study may indicate that the degradation of crude oil was enhanced by increasing the temperature of the system.[114]. However, without an understanding of the mechanism of the enhancement, the validity of the conclusion for other situations may be in doubt. The enhancement of degradation could have been induced by shifts in the members of the microbial community, changes in catabolic pathways by a microorganism, increases in enzyme reaction rates, or increases in the availability of the hydrocarbon substrates to the microorganisms due to physical

changes of the oil, including dispersion and emulsification. Also, the separation of biological degradation as a removal mechanism from abiotic loss mechanisms must be determined.

In order to accomplish bioremediation, the physical environment of the microorganisms responsible for the desired degradation must be conducive to their functioning. Microorganisms responsible for biodegradation may include indigenous microorganisms or microorganisms that must colonize the site in order to be active in the biodegradation process. An evaluation of the environment with respect to stress tolerable to indigenous microorganisms or with respect to conditions that allow colonization and maintenance of an active population of colonizing microorganisms is required to assess the potential for biodegradation. Critical environmental and biological factors that can be evaluated, and in some cases managed, for the enhancement of bioremedial processes include [17, 45]:

(1) Carbon source;

(2) Electron donors;

(3) Electron acceptors;

(4) Nutrients;

(5) Salinity

(6) pH;

(7) Temperature;

(8) Phase interfaces;

(8) Mixing and mass transfer;

(9) Solids (i.e., microorganisms) retention time (SRT);

(10) Concentrations of toxic or inhibitory compounds;

(11) Concentrations of contaminants; and

(11) Microbial populations.

2.7.1 Carbon Sources, Electron Donors, and Electron Acceptors

Non- halogenated organic compounds generally represent reduced forms of carbon,

making degradation by oxidation energetically favorable. An organic chemical is said to be reduced if it undergoes a net gain of electrons as a result of a chemical reaction (electron acceptor), and is said to be oxidized if it undergoes a net loss of electrons (an electron donor). Heterotrophic organisms (i.e., organisms that obtain their carbon from organic carbon, which includes humans and most bacteria, in contrast to autotrophs, which obtain their carbon from carbon dioxide in photosynthetic processes), oxidize organic compounds to obtain energy in a process called respiration. [196] In this process, electrons from the oxidizable organic compound (i.e., the substrate, which is the electron donor) are transferred to and reduce an electron acceptor. The electron acceptor may be an inorganic or organic compound. During this electron transfer process, usable energy for the organism is obtained through a complex series of oxidation-reduction (redox) reactions. The oxidation of organic compounds coupled to the reduction of molecular oxygen is referred to as aerobic heterotrophic respiration. When molecular oxygen is unavailable (i.e., under anaerobic, or more precisely, anoxic, conditions), the oxidation of organic compounds is coupled with inorganic or organic electron acceptors other than oxygen. Denitrifying bacteria can use nitrate (NO_3^-), sulfate-reducing bacteria can use sulfate ($SO_4^=$), while methanogens can use CO_2 as an electron acceptor in the production of methane. The potential energy available from the use of different electron acceptors varies, and a higher energy yielding process will predominate if the required electron acceptor is present.

In fermentation, which is a metabolic process that uses a series of enzyme reactions rather than the use an electron transport chain, an organic compound serves as both electron donor and electron acceptor, with a portion of the compound becoming a reduced end product and another becoming an oxidized product.[17] A common example of this process is the alcoholic fermentation of starch to CO_2 (the oxidized product) and ethanol (the reduced product). [196]

In an engineered bioremediation system, aerobic organisms will degrade biodegradable organic matter as long as oxygen is available. [17, 196] Electron acceptors tend to be used

successively in order of decreasing free energy yield. Therefore, if oxygen becomes depleted by the degradative process, and the other electron acceptors are present (i.e., nitrate, sulfate, and carbon dioxide), aerobic respiration will slow and eventually stop, while denitrifying organisms will become active and will use nitrate in the degradation of organic compounds until nitrate is depleted. Then sulfate reducers will become active as long as the sulfate concentration remains adequate, possibly leading to the production of sulfides. After depletion of sulfate, methanogens will form methane from acetic acid or carbon dioxide. Other electron acceptors such as iron or manganese may also be important in some anaerobic environments.

Anaerobic treatment using CO_2 as the terminal electron acceptor (referred to anaerobic digestion) has been used extensively for the treatment of biological sludges produced in wastewater treatment plants. Anaerobic digestion is dependent on three stages (i.e., three metabolic groups, or consortia, of microorganisms) to accomplish biodegradation. [187] In the first stage, hydrolytic and acidogenic bacteria hydrolyze organic compounds to organic acids, alcohols, CO_2, and H_2. The second metabolic group, called the H_2-producing acetogenic bacteria, converts the various products formed by the first group into H_2, CO_2, and acetate.The third group involves the bacteria that utilize H_2, CO_2, and acetate in the production of the final products CH_4 and CO_2. The range of organic compounds that can be broken down by anaerobic digestion is large, and includes carbohydrates, proteins, lipids, and petroleum hydrocarbons such as benzene, toluene, styrene, naphthalene, acenaphthalene, and benzothiophene.[83, 88, 90] Methanogenic degradation of aromatic hydrocarbons usually is dependent on an acclimation period, during which time the microorganisms develop a capacity to degrade the compounds.

Certain classes of compounds are degradable under specific redox conditions. For example, degradation of aliphatic hydrocarbons has not been reported without the presence of oxygen; oxidation of monoaromatic compounds have been demonstrated under denitrifying conditions[4, 164]; oxidation of toluene and xylene has been demonstrated under sulfate-reducing

conditions (though the accumulation of the reduced product hydrogen sulfide may inhibit the degradation process) [61, 62]; and an iron-reducing bacteria has been shown to able to degrade toluene, cresol, and phenol.[131] Summaries of laboratory research with regard to anaerobic transformation of hydrocarbons involving microcosms and enrichment cultures have been prepared by D. Grbic-Galic. [88, 89]

Halogenated organic compounds (i.e., compounds containing chloride, bromide, fluoride, or iodide ions), which are common contaminants of water sources and are particularly troublesome because of their low solubility, toxicity, resistance to both biotic and abiotic transformations, and their tendency to accumulate in food chains, are susceptible to anaerobic degradation, and especially degradation by reductive processes, rather than the oxidative processes that are more commonly responsible for the degradation of organic compounds. [184] Halogenated organic compounds are relatively oxidized by the presence of halogen substituents, which are highly negative, and thus are more susceptible to reduction. With increased halogenation, organic compounds become more likely to be reduced than oxidized. [214]. In the reductive process, which occurs in anaerobic environments, halogenated compounds can lose halogens through a process called reductive dehalogenation. Specifically, dehalogenation by reduction is the replacement of a halogen on an organic molecule by a hydrogen ion. Reductive dehalogenation is a cometabolic process, and an electron donor compound, such as lactate, acetate, methanol, or H_2, must usually be added to stimulate the process.[24] Since reductive dehalogenation results in compounds with lower numbers of halogens, these products are more susceptible to further degradation by oxidative and hydrolytic processes. Classes of compounds shown to be susceptible to reductive dehalogenation processes include: (1) carboxylated benzenes; (2) oxygen-, nitrogen-, cyano-, and methylene-substituted benzenes; (3) chlorinated benzenes; and polychlorinated biphenyls (PCBs).[120]

Anaerobic microbially-mediated reductive dehalogenation was observed about 25 years

ago by C.E. Castro and N.O. Belser,[36] but has been intensively studied for only about a decade.[198] As a remedial process for contaminated water sources, it is a new concept and still subject to field demonstrations. Research is currently being performed to better define the basic mechanisms of reductive dehalogenation reactions, especially with regard to developing anaerobic microbial systems with faster dechlorination rates as well as evaluating the potential for applying the process to bioremediation. [184] A possible obstacle to the use of reductive dehalogenation is the formation of halogenated intermediate products that may themselves be of public health concern. [138] Research studies have shown complete dehalogenation of some halogenated compounds [56, 72], but additional research is required to determine how effectively reductive dehalogenation can consistently reduce the levels of halogenated compounds to regulatory limits as well as result in the formation of non-toxic end products in complex water environments. Research areas that must also be addressed include: (1) methods to stimulate desirable metabolic sequences in contaminated systems through the intentional introduction of suitable electron donor and acceptor combinations [197] (for example, acetate has commonly been added as an electron donor in research studies [25]); (2) identification of levels of nutrients required to meet the nutritional requirements of dehalogenating microorganisms [157]; (3) identification of environmental factors and metabolic requirements that will result in complete reductive dehalogenation to non-toxic end-products [24]; (4) use of engineered microorganisms with optimum dehalogenating activity [157]; (5) development of cell-free enzymes capable of catalyzing reductive dehalogenation reactions [55]; (6) evaluation of rates of reductive dehalogenation processes with regards to meeting treatment objectives within regulatory limitations, since rates by indigenous microorganisms appear to be slow [24]; and (7) development of anaerobic microbial consortia that use reductive dechlorination for respiration rather than cometabolism, in order to increase transformation rates. [24]

Biodegradation potential of specific organic compounds is in part dependent upon the

aerobic/anaerobic status of the specific environment and the presence of specific electron acceptors. To assess the aerobic/anaerobic status of a contaminated water source or a treatment system, the redox potential, which reflects the potential for the transfer of electrons to a reducible material, can be measured by using a platinum electrode, or alternatively, by measuring oxidized-reduced couples of certain materials, such as ferrous ion (Fe^{+2}) and ferric ion (Fe^{+3}). The platinum electrode, which is sensitive and reversible to oxidation-reduction conditions, is used in combination with a reference electrode; measurements, referred to as Eh values, are reported in volts. Well-oxidized environments have redox potentials of 0.4 to 0.8 V, while extremely reduced environments may have potentials of -0.1 to -0.5 V[58]

2.7.2 Nutrients

Microorganisms also require an adequate supply of macro- and micronutrients for proper growth. Because many target chemicals are composed of a large percentage of carbon and low percentages of nitrogen and phosphorus, the rate and extent of biodegradation are often limited by low concentrations of nutrients in a water environment, and therefore nutrients must be added[52, 123]. Required macronutrients include phosphorus, nitrogen, and sulfur, while micronutrients (those required in minute quantities for growth) include many different substances, such as potassium, sodium, some metals (e.g., iron, magnesium, calcium, cobalt, potassium, molybdenum, and manganese), and vitamins (also referred to as growth factors). Required nutrients must be present and available to microorganisms in (1) a usable form; (2) appropriate concentrations; and (3) proper ratios. [58] Nutrient requirements of anaerobic microorganisms are generally lower than for aerobic organisms, because less biomass is formed. Requirements for nitrogen and phosphorus in anaerobic treatment processes have been determined to be in the C:N:P ratio of 700:5:1, compared to a recommended C:N:P ratio of 120:10:1 for aerobic treatment processes.

Bioengineers should attempt to determine which nutrients are required for a specific

process, as well as their optimal concentrations, though quantitative information is often lacking. Further research by microbial ecologists is required to determine nutrient requirements for different types of microorganisms that participate in biodegradation activities in order to be able to better control and predict the bioremedial process.

Another approach to the optimization of microbial requirements is use of a thermodynamic engineering modeling approach for the determination of the appropriate amounts of nutrients, electron acceptors and electron donors that must be supplied for growth of bacteria as well as for the estimation of the amounts of biomass and other products that will be formed. In a model developed by Dr. Perry McCarty of Stanford University[137, 142], calculations are made in which electrons from an electron donor are coupled with an electron acceptor to generate energy or used to synthesize biomass. The relative amounts of electron donor being oxidized for energy and being converted to biomass is established with an energy balance. The amount of energy released during oxidation of the electron donor must balance the amount of energy required to synthesize cell material.[24]

2.7.3 Salinity

Increased salinity in water sources can adversely affect the growth and activity of microorganisms.[114] Each microbial species has a limited range of osmotic pressures that it can tolerate. Species tolerant to high concentrations of solutes, referred to as halophiles, are able to tolerate higher salinity levels because they have either developed the ability to synthesize enzymes that function normally only at high temperatures, or are able to raise their internal osmotic pressure with internal solutes in order to balance the osmotic pressure of the external environment.[26] Fungi are generally more able to tolerate highly saline environments, including exposure to non-aqueous phases.

2.7.4 pH

Most microorganisms require a neutral or near neutral pH for optimal growth, though

specific microorganisms have their own optimum pH range. Aerobic microorganisms usually tolerate a wider range of pH than anaerobic microorganisms. [22]

pH and redox potential in some systems may jointly affect the extent of mineralization of organic compounds, since protons are often involved in biological oxidation/reduction reactions. For example, the mineralization of naphthalene and octadecane in sediments was shown to be enhanced by higher redox potentials and higher pH values, suggesting that aerobic microorganisms in the upper, oxidized sediments possessed the greatest potential for mineralization of these reduced substrates. [92]

2.7.5 Temperature

Optimal temperatures for biodegradation may vary, according to the specific process. In conventional aerobic treatment, the optimal temperature ranges from 20° C to 25° C. For mesophilic anaerobic treatment, the optimal temperature is 35° C, and for thermophilic bacteria, the optimal temperature is 55° C. Also, biological processes exhibit a temperature dependency. An exponential increase in reaction rate is observed up to the optimum temperature, or temperature range. Above that temperature range, the reaction rate rapidly decreases. [98]

2.7.6 Phase Interfaces

Phase interfaces have been recognized as sites of enhanced biodegradative activity. [114] At these interfaces, higher concentrations of contaminants are often found. With liquid-gas interfaces, such as are found in lakes and ocean waters, compounds accumulate in the liquid surface micro-layer. The addition of inert solids to bacterial cultures and water environments also results in enhanced biodegradation and increase in cell numbers. Microbial cells naturally adhere to and colonize virtually any surface immersed in an aquatic environment. [65] The tangled mass of cells that develop on a surface are referred to as a biofilm. These liquid-solid interfaces not only provide surfaces for the attachment of microorganisms, but also ion-exchange and adsorption sites for contaminants, microorganisms, and their enzymes. Adsorption of contaminants to solids

may protect them from biodegradation, or conversely, degradation may be enhanced if contaminants are sorbed to a surface that is readily colonized by microorganisms. In some cases, compounds have been shown to be sorbed to the surfaces of microorganisms without the metabolic capability to degrade them; this sorption often results in protection from degradation by other microorganisms.[220] Biodegradation may also be enhanced if toxic metabolic products are removed from the environment by sorption to solids, and if the pH of the environment is regulated by the buffering capacity of the solids.

Liquid-liquid interfaces, such as in mixtures of oil and water, are often present in contaminated environments. Biodegradation of components and contaminants of the oil are limited by their solubility in water, since microorganisms usually inhabit the water phase of an environment. Microorganisms can become associated with the oil-water interface and accomplish biodegradation at this interface. Most microorganisms can not survive in the oil phase; those that can must obtain water and water-soluble nutrients at the oil-water interface.

2.7.7 Mixing and Mass Transfer

Adequate mixing is required for the transport of nutrients, electron donors, electron acceptors, and any other required amendments to microorganisms responsible for biodegradation. Inadequate mixing in bioreactors may lead to system failure, even if all other environmental requirements are met. Bioremediation of ground water is especially challenging in terms of ensuring contact among contaminants, microorganisms, required nutrients and other amendments.

Oxygen mass transfer is often an important limiting factor in the overall reaction rate in waste treatment processes. Oxygen mass transfer can be increased by: (1) increasing the saturation concentration by increasing total pressure or by increasing oxygen partial pressure; (2) increasing the concentration gradient by increased mixing or decreased diffusion distance; (3) increasing the area of gas-liquid interface, by using small bubbles or high turbulence; (4) increasing the diffusion coefficient by increasing temperature; or (5) using oxygen substitutes such

as hydrogen peroxide.[98]

2.7.8 Solids Retention Time

Solids (i.e., microorganisms) retention time (SRT) is a measure of the average length of time microorganisms spend in a bioreactor. If environmental requirements are met, longer retention time of microorganisms in bioreactors results in greater biodegradation. [17] SRT is evaluated as the mass of microorganisms in the system divided by the mass of microorganisms removed (i.e., wasted) per unit time. Another important aspect of bioengineering of bioreactors is the difference between hydraulic retention time (HRT) and SRT. For continuous-feed, completely mixed systems without solids (i.e, microorganisms) recycling, SRT equals HRT. For continuous-feed, complete mix systems with recycling (or continuous-feed, fixed film systems), SRT may be many times greater than HRT. In an ideal system, HRT should be low and SRT should be high. Low HRT allows for greater feed flow rates in a bioreactor, but a high SRT will lead to more effective biodegradation of organic compounds, and allow a process to meet regulatory effluent quality requirements.

Growth and increase in biomass of microorganisms in a bioreactor can be modeled using biokinetic models. These models may be found in standard environmental engineering textbooks.[8, 14, 50, 145, 159]. One model commonly used for aerobic systems is the Monod model.[17] The Monod expression assumes a first-order relationship between substrate concentration and biomass conversion. The equation may be modified to allow for competition between substrates or for limiting nutrients.[139]

Using the Monod model, four kinetic parameters, which can be used to bioengineer a bioreactor system, are determined experimentally. [17] These parameters include: (1) k, maximum rate of substrate use per unit weight of microorganisms, (2) K_s, Monod half velocity coefficient, equal to the substrate concentration when the rate of microbial substrate use per unit volume is equal to 0.5 k, (3) Y, growth yield coefficient, and (4) b, microorganism decay coefficient. Each

organism has a characteristic set of kinetic parameters, which cannot be changed by the bioengineer. However, when microorganisms undergo genetic changes, i.e., mutation, in response to exposure to toxic chemicals, these kinetic parameters can change. Bioengineers refer to these changes due to mutation as acclimation. They describe this phenomenon of acclimation as the development of resistance to toxicity or the development of a mechanism (e.g., the production of an enzyme) that leads to enhanced biodegradation. Basically, any indigenous microorganism is viewed as adaptable to and able to degrade any synthetic organic compound. However, further research is required to identify the conditions and factors that affect the ability as well as the amount of time required for microorganisms to acclimate to specific organic compounds. These factors may include substrate structure, co-occurrence and concentration of other more easily degradable substrates, and environmental conditions. Examples of the use of acclimation in bioremediation are given in Table 3.

The bioengineer uses the kinetic parameters in equations that provide useful information that can be used to optimize SRT and HRT in a bioreactor. [17] For example, the concentration of effluent substrate (i.e, waste organic constituent), the amount of sludge that will be produced, and the concentration of microorganisms at steady state in the bioreactor can be predicted with the parameters. With this information, the bioengineer can obtain the desired HRT by controlling the rate of influent, and obtain the desired SRT by selecting the amount of sludge to be wasted from a complete-mix system. With a high concentration of active biomass, the size of the bioreactor can be reduced, and the conversion of toxic compounds can be increased. [212] With a greater rate of degradation, concentrations of contaminants can be kept below toxic limits. High concentrations of organisms can be maintained by separation of microorganisms from treated wastewater and subsequent return to the bioreactor, by immobilization of microorganisms on carrier materials, or by the use of membrane reactors.

The bioengineer can only change the microbial growth that leads to the production of

sludge by selection of microorganisms.[17] Sludge minimization is accomplished by selection of the appropriate microorganisms. For example, anaerobic processes result in production of almost an order of magnitude less sludge than aerobic processes.

2.7.9 Toxic or Inhibitory Compounds

Treatability studies should be performed on any proposed biological treatment process for a specific contaminated water to determine if there are any organic or inorganic contaminants that may cause inhibition of the biodegradative processes. Inhibition may be non-competitive or alternatively, competitive, in which the substrate and the toxic, inhibitory compound compete for the same enzyme site. Many organic compounds, such as formaldehyde, can cause competitive inhibition, while inorganic compounds such as ammonia and nickel can cause non-competitive inhibition.[18, 19] During the degradation process, intermediate products may be formed that are also toxic to the bioprocess and may have to be removed. In complex systems, if two substrates are present in high concentrations, the more easily metabolized substance can repress and inhibit the metabolism of the other compound.

Again, using modelling techniques, bioengineers can determine an inhibition coefficient, K_I, which is a measure of microbial resistance to toxicity.[17] This coefficient cannot be changed by the bioengineer, because if an organism does not have the ability to resist toxicity, no engineered bioprocess can create that ability. However, the microorganisms may, through mutation, or acclimation, develop resistance, thereby increasing K_I. For example, carbon tetrachloride, a highly chlorinated solvent, has been shown to be toxic to unacclimated cultures of anaerobic microorganisms at 0.5 mg/L, but with acclimation, 15 mg/L could be tolerated. [227] Results of other studies have indicated that in general, the maximum allowable concentration for treatment of chlorinated compounds ranges from between 10 and 100 mg/L, depending on the specific compound. This range is typical of levels found in many contaminated ground water systems.[24] Other organic substances that are inhibitory include alcohols, phenols, agricultural chemicals,

organic nitrogen compounds, and surfactants. [194] These compounds may serve as substrates that are biodegradable at lower concentrations, but may be toxic at higher concentrations.

The most well-known group of toxic substances that can inhibit biological processes are the heavy metals.[194] This group includes many transition elements (e.g., cadmium, chromium, copper, mercury, nickel, zinc), some non-transition metals (e.g., lead), and the metalloids (arsenic and selenium). In anaerobic systems, metals are toxic to anaerobic organisms at very low concentrations. Toxicity seems to be associated with free metal ions, however, so the degree of toxicity is dependent on the presence of complexing or precipitating anions. Metal sulfides are extremely insoluble, so if contaminated water contains high levels of sulfur compounds (e.g., 0.5 mg sulfide/mg toxic metal), fairly high concentrations may be tolerated in the water.

As more information is developed concerning environmental factors that enhance acclimation, bioengineers may be able to provide conditions to encourage acclimation, thus resulting in increased resistance to toxicity as well as increased biodegradative capabilities. [17] One technique for the development of microorganisms for the degradation of specific compounds is by identifying organisms shown to be active in the presence of a specific toxic compound, adapting them to progressively higher concentrations of the compound, selecting the most active colonies, and preserving them for later use in bioremedial systems. This technique is a type of genetic engineering process, which has been used to produce microorganisms that are acclimated for the degradation of a specific organic compound. This technique involves the selection and breeding of a clone from a single organism that exhibits some type of desirable properties.The desirable properties available are those related to the natural variability of the group of microorganisms investigated.

2.7.10 Concentration of Contaminants and Cometabolism

Biodegradation of a contaminant being utilized by microorganisms as a primary carbon source is controlled by a limiting concentration of that contaminant (e.g., < 50 ppm). Below that

concentration, referred to as S_{min}, microorganisms cannot use the contaminant, because the concentrations may be too low to stimulate production of the specific enzymes required to degrade the contaminant.[17] S_{min} is determined by the four kinetic parameters from the Monod model, and cannot be engineered by the bioengineer. The bioengineer can, however, select microorganisms that have a set of kinetic parameters that will lead to a low S_{min}.

Biodegradation of organic compounds with concentrations lower than S_{min} can be accomplished if the microorganisms can use the compounds as secondary substrates, or cometabolites.[17] Cometabolism is the process of degrading compounds without metabolizing them, i.e., the compounds are not consumed as a source of carbon or energy; biodegradation of the compounds does not lead to energy production or cell growth.[106] Cometabolism has also been referred to as co-oxidation if the transformation involves an oxidation reaction.[160] During metabolic activities, enzymes are produced for use in degrading specific primary substrates, but these enzymes can also initiate the degradation of a range of nonspecific compounds that are neither essential for, nor sufficient to, support microbial growth. These non-growth, or secondary, substrates are usually only incompletely oxidized, or otherwise transformed, by the microorganism involved, although other microorganisms may utilize by-products of the cometabolic process. For cometabolism to occur, a primary substrate(s) must be present for the microorganisms to use a carbon source. For example, in municipal wastewater treatment plants, domestic wastewater may serve as the carbon source, and low concentrations of organic compounds in the wastewater may be biodegraded by cometabolism by the existing microorganisms in the treatment plant.

An advantage to cometabolic degradation of a contaminant is that there is no lower limit to its final concentration.[213] If a contaminant served as a primary substrate, fewer microorganisms would survive when its supply became low, and further degradation would cease.[136] However, when a contaminant is used as a secondary, cometabolic substrate, it can be continued to be

degraded by a large, healthy population grown on the primary substrate until the contaminant is completely transformed.

Cometabolism may be a prerequisite for the mineralization of many recalcitrant substances found in the environment, such as polynuclear aromatic hydrocarbons. [114, 115] Another example of the cometabolic process is the cometabolism of halogenated aliphatic compounds, such as trichloroethane (TCA), using methane as the primary substrate. [45] Methanogenic microorganisms cometabolically degrade the halogenated compounds, forming transformation products. Other heterotrophic bacteria continue the degradation to stable end products. Cometabolism is found both under aerobic and anaerobic conditions.

The underlying mechanisms that result in cometabolism have not yet been clearly elucidated. Possible mechanisms that have been postulated include [114]: (1) the non-growth substrate is unable to act as an inducer of the pathway(s) required for its own transport into the cell or for its metabolism, but the primary substrate can; (2) the structure of the non-growth substrate prevents its metabolites from acting as nutrients; (3) the non-growth substrate or its metabolites are co-repressors for a growth-limiting cellular function, but the primary substrate relieves the repression; and (4) growth on the other substrate may provide energy required for the cometabolism.

2.7.11 Microbial Populations

An appropriate and active population of microorganisms must be present in a bioremedial system to accomplish biodegradation of specific organic compounds. Microorganisms have outstanding capabilities to degrade organic molecules. Almost all naturally-produced organic compounds, including those with such substituents as halogen atoms, are degradable by some microorganisms or consortia of microorganisms; this versatility extends to anthropogenic compounds also. It has been said that microbes are infallible with regard to their ability to degrade organic compounds, i.e., whatever man or nature can make, microorganisms can

degrade.[194] This ability may be expected for naturally occurring compounds, since it would involve a reversal of existing bio-synthetic pathways, but for xenobiotic compounds, i.e., those that do not occur naturally, microorganisms may not have the enzyme systems required. However this principle appears to be true to a considerable extent, and it would be difficult to disprove. Examples of classes of compounds that contribute to serious pollution problems in water environments and that been demonstrated to be biodegradable (to some extent) include: chlorinated phenols, haloalkanes, nitroaromatic molecules, chlorinated biphenyls (PCBs), chlorinated phenoxy herbicides, chlorobenzenes, aromatic and polynuclear aromatic hydrocarbons (PAHs), and pesticides such as the triazines, organophosphorus compounds, carbamates, anilines, and pyridines. In the Netherlands, a list of 140 groups of organic compounds have been identified having environmental significance; of those 140 groups, 75 percent have been identified as being able to be completely or partially degraded by microorganisms under aerobic conditions, while 30 percent have been shown to undergo anaerobic biodegradation. [224]

The ability to detect and isolate microorganisms with specific metabolic capabilities in the environment is being developed with a procedure called gene probing. [173] To prepare a specific probe for a specific metabolic capability, DNA of known metabolic origin is isolated, purified, and labelled (e.g., with ^{32}P, ^{3}H, ^{35}S, antigen-antibody complexes, or enzyme-substrate reactions). Then the double helical structure of the probe DNA is destroyed by heating to create single-stranded probe nucleic acid. The probe is then added to the sample of interest, in which target DNA from a bacterial colony or an extract of DNA from an environmental sample has been similarly treated as the probe DNA and bound to a hybridization filter. Under proper conditions, the probe and target DNA are allowed to re-associate to re-form the double helical structure. Re-association of the probe with complementary strands of the target nucleic acid results in a hybrid molecule that is readily detected by the probe-associated label.

Gene probes have been used to monitor and detect specific degradative genotypes in complex environmental matrices, as well as to investigate the stability of specific genotypes with specific metabolic capabilities over time. Also, mineralization rates of specific organic compounds have been correlated with genotype frequency over time. In a study by J.W. Blackburn, R.K. Jain, and G. S. Sayler,[21] the population density of a genotype of microorganisms that can degrade naphthalene in an activated sludge system was monitored using a gene probe specific for naphthalene-degraders. An increase in cell numbers of the naphthalene-degrading genotype was directly correlated with an increase in mineralization of naphthalene and a decrease in removal of naphthalene by volatilization. Results of such a study may be used to provide information that can be used to determine bioengineering activated sludge treatment process variables that will optimize naphthalene-degradative microbial cell densities at levels that will result in the maximum biological mineralization. The use of gene probes to determine critical cell densities and to control system variables should be encouraged for a variety of treatment systems, in order to enhance naturally occurring biodegradation or to establish engineered biodegradative processes. [173]

To achieve biodegradation, the following potential uses of microbial populations should be considered: (1) microbial consortia; (2) fungi; (3) immobilization of microbial populations; (4) bioaugmentation using natural or genetically engineered microorganisms; and (5) genetically engineered microorganisms.

2.7.11.1 Microbial Consortia

In an effective bioremediation process, usually a consortium of several types of microorganisms are present that together interactively accomplish mineralization of the organic compounds.[114] For example, the interactions that occur in a consortium may involve partial transformation of a substrate by one microbial group, with subsequent utilization of the transformed product by a second group. The second group may excrete some growth factor essential to the first group or may remove the product of the metabolism of the first group, if it is

inhibitory to the first group, resulting in mutual positive feedback between the two groups. Other groups of microorganisms may also be present in the consortium, utilizing transformation products of the first two groups. The advantages of microbial consortia include [196]: (1) thermodynamically unfavorable reactions are made possible when metabolically linked with favorable reactions within the consortia; (2) toxic or inhibitory compounds are degraded by resistant members of the consortia; and (3) newly introduced contaminants are degraded more quickly than if a given species had to evolve a novel complex degradation pathway.

2.7.11.2 Use of Fungi

In most bioremedial processes, bacteria are the primary types of microorganisms that accomplish degradation of specific organic compounds. Recently, research has focused on the use of fungi in bioremedial systems. Fungi are part of the natural scheme of carbon recycling, but have not been extensively used for the treatment of wastes. Many fungi that are found in waste treatment systems are pathogenic, while others cause a disruption in the treatment process (e.g., in activated sludge systems, they can cause precipitation of the sludge blanket). However ,wood-degrading fungi are currently being investigated for their potential to degrade hazardous wastes.[6, 33, 81] The white-rot fungi can degrade lignin, which is one of the most recalcitrant natural molecules, and which is thought to be similar in structure to aromatic organic waste compounds. Therefore, theoretically, this fungus, *Phanerochaete chrysosporium*, should be able to degrade aromatic hazardous materials. The fungus operates in two distinct metabolic cycles. In the primary, or growth, cycle, the fungus utilizes carbon substrates such as sugars or polymeric saccharides such as cellulose. However, depending on growth conditions and the availability of nutrients such as nitrogen, the fungus may adopt a secondary metabolic cycle in which the organism secretes a complex mixture of peroxidases commonly referred to as ligninases, which are used in the degradation of lignin. The ligninases rely on a supplemental enzyme system to supply the necessary hydrogen peroxide to initiate the oxidation of lignin. Lignin has a random composition and a highly polymeric structure, so the enzymes that degrade lignin have low

specificity, meaning that they can react with many substances, including organic waste compounds.

Research is being conducted on the use of the white-rot fungus for the degradation of organic contaminants associated with the wood-preserving industry. [82] A biological reactor, incorporating the necessary conditions for the cellular morphology of fungi and the required physiological requirements, has been developed for the treatment of liquid waste streams. The reactor is configured as a rotating biological contactor (RBC), consisting of several rotating disks on which the fungus can attach and grow. The fungus is sensitive to shock loadings, which can reduce its degrading ability, and use of the RBC protects the fungus from these effects. Bench scale tests using the RBC have shown that pentachlorophenol can be degraded from 250 mg/L to 5 mg/L in eight hours. Further research is being conducted to determine optimal operating conditions, including mass transfer of oxygen and substrate to the fungi and type and quantity of growth substrates required.

2.7.11.3 Immobilized Microorganisms

Immobilization of microbial cells has been used to ensure the presence of appropriate microorganisms in an engineered bioremedial system, which in turn will enhance the biodegradative potential of the system.[51, 60, 65, 116, 181, 191, 195, 218] Immobilization of microbial cells refers to the transfer of the cells from a free state to a state in which they are confined or localized in a defined region of space, with retention of catalytic activity, and if possible, with retention of viability so that the cells can be used continuously or repeatedly.[65] Various gel compounds and other materials are used to entrap the microbial cells. Immobilized cells can conduct multi-enzyme reactions as easily as free cells, because, in their immobilized state, they are present in a much higher initial biomass concentrations (with concentrations of greater than 1010 cells per mL of matrix possible), resulting in faster reaction times than with free cells. Natural "biofilms" also are considered to be immobilized cultures, usually depending on polysaccharide

gel secretions for entrapment.

Strains of microorganisms selected for metabolic capability for the degradation of specific compounds are immobilized in matrices such as polysaccharides (e.g., alginate and carrageenan), polyacrylamide, polyurethane foams, and activated carbon. [65] The selected bacteria are grown in large quantities in fermentors, concentrated by filtration or centrifugation, and entrapped in the immobilization matrix. These immobilized cells are usually used in some type of bioreactor, such as a batch, fluidized-bed, or packed-column reactor. Immobilized cells have been shown to be long-lived and able to tolerate concentrations of toxic chemicals that would kill free cells. Their high biomass densities in the immobilized cells result in high total biodegradative activity. Immobilized cells have characteristically long periods of enzyme activity, though eventually the immobilized cells show a loss of activity. In some cases, inactive matrices can often be re-activated by incubation under appropriate conditions. One especially desirable use of immobilized cell technology is the ability to customize treatment for particular types of contaminated waters by mixing supports containing different pollutant-degrading bacteria immobilized separately or together. [51]

One of the major problems associated with the use of immobilized cultures is the transport of target contaminants into the immobilization matrix. [65] This transport is limited by a double-diffusion gradient that builds up: one into the matrix (i.e., through the matrix-liquid interface) and the second from the matrix to the cell (i.e., through the matrix-cell membrane interface). As a result of these diffusion barriers, many micro-environments within the matrix can be created, with differences in pH, oxygen concentration, and concentrations of substrate and transformation products. In some cases, a permeabilizing reagent may be added to the immobilized cells to decrease the diffusion resistance, which, however, may result in the death of the cells, though enzyme activity will be retained. The use of a too permeable structure may result, however, in excessive leakage of cells and required enzyme cofactors.

Immobilized cells usually become concentrated at the surface of the matrix, while cells within the matrix, due to problems with diffusion of oxygen and nutrients into the matrix, may lose their viability or may lyse.[65] However, as outer layer cells die and the outer layers of the matrix begin to disintegrate, diffusion to the inner cells may result in the inner cells becoming viable again as the barriers to diffusion of oxygen and nutrients are overcome. Cell lysis may also become a problem if the substrate or a transformation product is toxic to the cells, especially if these toxic substances are slow to diffuse away.

The immobilized matrix may also be destroyed by simple physical abrasion, which causes tears and breaks in the structure. [65] In addition, cell division and growth, with resulting carbon dioxide production, may result in the breakup of the immobilized matrix. This problem may be controlled by supplying cells with only enough nutrients to keep them in a resting but viable state. Maintenance of cells in a resting state ensures that cofactors and other essential enzymes are continuously regenerated, but does limit the selection of microorganisms to those with enzymes that are always present in the cells (i.e., constitutive enzymes) rather than inducible enzymes, so that the desired degradative activities are carried out. Side reactions that do not result in degradation of the target chemicals may also occur, since immobilized cells have many metabolic pathways; these reactions may result in disruption of the matrix if carbon dioxide is produced in large amounts.

The use of immobilized genetically engineered microorganisms may be more effective than the use of free genetically engineered microorganisms because, since immobilized cells are often in a non-growth state, there is no loss of plasmids, which is unlike the loss that can occur in actively growing cells.[65] Possible applications of genetic engineering to improvements in the use of immobilized cells include: (1) development of strains that over-produce the required enzyme so that the majority of the energy in the system is used to degrade the target compounds rather than used in other, less desirable side reactions; and (2) development of strains in which inducible

enzymes are converted into constitutive types by alteration of the regulatory mechanism of the cell, so that prior induction of enzymes is not required.

An alternative to the use of immobilized whole cells is the use of immobilized enzymes[93] Based on the techniques developed for the preparation and use of enzymes in industrial applications (e.g., immobilized lactase in the milk industry; glucose isomerase in the sugar industry; aminoacylase in the production of food supplements, medicines, and cosmetic additives), applications for the treatment of wastewaters have been proposed. Many enzymes for the degradation of many organic waste constituents have been identified; these enzymes could be used in waste treatment systems in immobilized states. For example, parathion hydrolase, covalently immobilized on glass, has been shown to able to detoxify organophosphate pesticides in industrial wastewaters[149]; immobilized parathion hydrolase has also been shown to degrade parathion to *p*-nitrophenol and diethyl thiophosphoric acid [200]; immobilized phosphotriesterase has been used to detoxify organophosphate pesticides[34]; and immobilized peroxidase may potentially be used to remove carcinogenic aromatic amines such as benzidine, naphthylamine, and aminophenyl found in wastewaters from the coal, plastic, and textile industries.[117]

2.7.11.4 Bioaugmentation

Bioaugmentation, commonly referred as microbial seeding or microbial inoculation using acclimated or genetically engineered microorganisms, has been used as a means of treating contaminated water sources. Treatment testing of bioaugmentation has generally been performed without the use of adequate experimental controls to demonstrate that improvements were due to the addition of the microbial inoculant, rather than due to other uncontrolled factors, and without the use of a chemical mass balance approach to the assessment of fate and transport of the target chemicals.[70] In addition, treatment results at field scale have been generally presented without a critical evaluation by experts (i.e., without peer review). Without the use of adequate scientifically controlled studies, determination of whether these products are beneficial, detrimental, or

irrelevant to treatment is often not possible.

Difficulties are incurred by introduced microorganisms as they try to function in a contaminated environment. The introduced microorganisms may be subject to predatory grazing by other organisms, such as protozoa, which have been identified as a major factor regulating bacterial populations. [2, 91] In addition, microorganisms introduced into a non-sterile environment will experience competition for those factors essential for its survival and activity. These factors may include energy sources, essential nutrients, space, and attachment sites. Also compounds rarely occur singly in a contaminated environment; other compounds present may have beneficial or inhibitory effects on the introduced microorganism. A strain introduced at one site may be effective in the degradation of a target compound, but at another site, due to environmental conditions, be unable to degrade the same target compound. The ability to predict whether a specific microorganism will become established in a specific environment has not yet been developed, because of the many interdependent physical and biological factors that impact upon the ability of a microorganism to become established and to function in an environment. More research is required to identify the barriers to colonization by introduced microorganisms and to develop engineering responses to those barriers.

Research is also required to identify potential ecological impacts of introduced microorganisms in different environments. [192] For example, introduced microorganisms could potentially displace indigenous microbial species vital to the operation of the local ecosystem.

Microbial seeding using a microbial consortium may be a more effective means of bioaugmentation than introducing a single microorganism to an environment. Seeding a contaminated water source with members of a consortium previously selected for the degradation of a specific target compound may result in acceptable degradation, provided that each member can survive in the physical environment present at the site, has access to the contaminant, and can utilize or tolerate the concentration level of the contaminant. Microbial seeding is also more likely to succeed in bioreactor systems rather than in *in situ* systems, since environmental conditions in

bioreactors can be more easily controlled.

2.7.11.5 Genetic engineering

A process called genetic engineering is a recent development of the field of molecular biology. Genetic engineering allows the manipulation of the genotype of microorganisms, which has led to the ability to manipulate genes *in vitro*. Advances made with new techniques in genetic engineering include the ability to isolate DNA from any source and to introduce it into organisms for which procedures for gene manipulation have been developed.[114] This technique involves the following three steps[115]: (1) insertion of the selected DNA into a special DNA molecule, the vector, which is used to transfer the selected DNA into the new host (commonly used vectors are plasmids, which are small circular extrachromosomal DNA molecules that can replicate autonomously in the host cell); (2) transfer of the modified vector into the host bacterium in such a way that is can be replicated and expressed; and (3) identification of the host bacterium that has taken up the modified vector and separation of this colony from unmodified colonies.

A genetically engineered microorganism introduced to an environment may respond in the following ways[114]: (1) the microorganism will be unable to reproduce but will be metabolically active; it may, however, be able to transfer its DNA to other microorganisms by conjugation or transduction; (2) the microorganism will be able to reproduce, but will be unable to establish a stable population; it may transfer its DNA to indigenous microorganisms, leading to a persistence of a genetic potential in the environment; or (3) the microorganism will be able to both reproduce and persist indefinitely in the environment. Instability and loss of genetically engineered microorganisms can occur in several ways, including [114]: (1) loss of the plasmid during replication of cells, resulting in a loss of key activities; (2) dilution of the microorganisms in the system due to increases in plasmid-free microorganisms; and (3) replacement of the microorganisms by indigenous microorganisms. Such problems may be minimized by using massive inoculum of the genetically engineered microorganisms or by using immobilized cell

systems, allowing the retention of high concentrations of organisms in the bioreactor. However, even if the host cells do not survive, the altered DNA fragments may be passed on the other microorganisms in the environment that are better adapted to growth within the particular environment. This transfer can be encouraged if the DNA is placed within a highly conjugative plasmid.[114]

Applications for use of genetically engineered microorganisms are focusing on several different aspects of waste treatment. These applications include[15]: (1) optimization of existing processes, such as improved tolerance to extreme environmental conditions and increased resistance to inhibition by toxic substances; (2) development of new processes, i.e., the development of new metabolic pathways; (3) enhancement of degradation rates, e.g., by enhancement of enzyme production; (4) increase in extent of treatment, i.e., lower final concentrations of pollutants; (5) utilization of multiple substrates simultaneously; and (6) improvements in substrate uptake mechanisms. B.E. Rittmann[167] and J.B. Johnston and S. G. Robinson[114] have identified several specific improvements in the biological treatment of waste waters that may be possible with the use of genetically engineered microorganisms, including (1) elimination of activated-sludge bulking (i.e., improved flocculation and settling); (2) reduction in sludge volume; (3) improvements in biofilm attachment; (4) reduction in oxygen limitations in aerobic processes; (5) enhancements in the biodegradation of xenobiotic organic compounds; (6) resistance to toxic upsets; (7) increased stability of anaerobic digestion processes; and (7) enhancement of sludge dewaterability. These improvements might be accomplished by modifications in enzyme activity, including[14]: (1) increase in enzyme levels in a microorganism; (2) re-arrangement of regulatory DNA base sequences controlling the expression of specific genes in response to specific stimuli; (3) introduction of genes for new enzymatic functions into microorganisms that do not normally have those functions; and (4) modification of individual genes to alter the characteristics of individual enzymes, such as substrate specificity, kinetic

parameters, or environmental requirements. Advances in genetic engineering at the laboratory scale for water cleanup are summarized for the degradation of a variety of pollutants by M. Alexander[1], G.S. Omenn and A. Hollaender[156], J.B. Johnston and S. G. Robinson[114], G.S. Sayler and others [174], A.M. Chakrabarty[41], H-J. Knackmuss[118], the Hazardous Materials Control Research Institute [95], Rubio and Wilderer [170], and R.R. Fulthorpe and R.C. Wyndham[75].Specific examples of the use of genetic engineering for bioremediation are given in Table 4.

In certain highly contaminated *in situ* aquifer environments, microbial seeding of genetically engineered microorganisms has been proposed as a possibly effective means of bioremediation. A microorganism could be constructed to be able to transform and detoxify a target contaminant, but also would be constructed so as not be able to grow and divide under the environmental conditions present at the site. For example, the microorganisms could produce large amounts of the enzymes responsible for transforming the target contaminant, but the enzymes would not provide benefits to the microorganism itself. The microorganism could be engineered to use a specific substrate as an energy source that could not be used by the indigenous microorganisms; this energy source would be supplied to the contaminated environment. Chemical agents could be used to temporarily inhibit indigenous organisms to enhance the competitive ability of the introduced genetically engineered microorganism to survive. The regulatory aspects of such a scenario have not yet been decided, but this example illustrates some of the applications of genetic engineering that have been proposed for the enhancement of bioremediation.

To select microorganisms appropriate as a source for biodegradation applications of genetic engineering, the following criteria have been suggested[114]: (1) the microorganisms should be normally present in soils, sludges, water, sources, or wastewaters; (2) they should be tolerant of extreme or toxic environments; and (3) they should be known to possess either a wide range of biodegradative capabilities or the capability for a specific biodegradative pathway of importance.

Types of biodegradative pathways useful for remediation include the capacity to metabolize long chain or branched alkanes; degradation of polycyclic aromatic hydrocarbons, and the ability to perform dehalogenation reactions. In addition, the microorganisms should be non-pathogenic to humans, plants, or animals. At this time, a major limitation in the use of genetic engineering in pollution management is that most of the bacterial genera known for the biodegradation of environmental pollutants are not yet well characterized in terms of gene exchange. [114] Fungi may also serve as a useful source of genes for metabolic enzymatic activities because of their tolerance of hostile environments and their ability to degrade polymers. [114] However, gene manipulation within fungi are complicated by their sexual and asexual reproductive methods as well as lack of well-developed techniques for introducing specific segments of DNA into fungal cells.

Another potential problem with the use of genetic microorganisms in the field of waste treatment is the inability of the genetically engineered microorganisms to compete with indigenous organisms present in the contaminated water or reactor system, which are non-sterile. Selection of an appropriate genetically engineered microorganism should be conducted in relation to the ecological constraints of the target environment or bioreactor, such as nutrient status or concentration of contaminants present, i.e., the microorganism should be able to function in the target environment. Also, since most biological treatment occurs as the result of a consortia of microorganisms in order to complete degradation of a specific organic substance, rather than as a result of only one type of microorganism, the use of one genetically engineered microorganism may not result in the degradation of the target compound.

The release of genetically engineered microorganisms is controversial and has stirred up considerable debate [63]. Therefore, the bioengineer must consider the potential hazards of release of genetically engineered microorganisms to the environment. Selection of a microorganism for genetic manipulation should not only include considerations of its degradative abilities but also include considerations of all known properties of the strain, including any pathogenic properties

or potential. The microorganisms must be chosen and handled in such a way that they represent no threat to health to workers handling them or to anyone who might subsequently contact them in the environment. There is no way to completely assure the containment of microorganisms and their DNA in full scale waste treatment systems. For example, microorganisms have been found in aerosols downwind from treatment plants. Complete sterilization of effluents would be difficult to perform, for no disinfectant is completely effective; economic constraints would also be limiting. The possibility of transmission of chemically intact DNA exists, even from dead microorganisms, though it has not yet been shown to occur in natural environments. Therefore, any use of genetically engineered microorganisms for waste treatment should be considered and accepted as a deliberate and irreversible release of the microorganisms to the environment. The impacts of this release need to be thoroughly understood before the use of genetically engineered microorganisms is permitted. The use of genetically engineered microorganisms for environmental applications is regulated under the Toxic Substances and Control Act (TSCA). [119] Any application of genetically engineered microorganisms for a specific bioremedial process should be carefully investigated and monitored using guidelines developed under TSCA.

In addition, the California Biotechnology Council [192] has suggested that research monies should be used to focus on finding indigenous microorganisms that can degrade specific organic chemicals, since natural microorganisms have been shown to have a wide ability to degrade organic chemicals, and they will also be native to the habitat, rather than spending large amounts of money engineering unproven, risky "superbugs".

3.0 REGULATORY ISSUES

3.1 Legislation Regulating Bioremediation

Bioremediation of a specific water environment is regulated by legislation that addresses either the use of the water or the history of its contamination. [50, 54] Passage of the Safe Drinking Water Act in 1974, and the Safe Drinking Water Act Amendments (SDWA) of 1986 increased

requirements for removal of pollutants from drinking water, including chemical, biological, and particulate contaminants for the approximately 240,000 public water supply systems serving 170 million people. According to the U.S. Environmental Protection Agency, more than half of these systems are out of compliance.[54] Applications of bioengineering systems in the treatment of drinking water for meeting new standards have been described for the removal by slow rate sand filtration of organic chemicals [47, 48] and particles [144], where the degree of treatment was related to amount and type of biomass. Biologically active rapid filters have been developed to remove mono- and di-chlorophenols and mono-and di-chlorobenzenes, [132] trihalomethane precursors and organic carbon, [124] as well as iron and manganese. [148]

The Clean Water Act is directed at used water in the form of municipal and industrial wastewaters. A list of toxic pollutants was developed (i.e., the priority pollutants), and effluent guidelines for point sources of wastewater were promulgated by the U.S. Environmental Protection Agency as the National Pollutant Discharge Elimination System (NPDES), which identified standards of performance for treatment. Under the Water Pollution Control Act Amendments of 1981 (Public Law 97-17), bioengineering systems were approved to accomplish secondary treatment; these systems included oxidation ponds, lagoons, and trickling filters.[50] These bioengineering systems were effective for removal of general organic compounds at about 85% removal efficiency. The use of the activated sludge process for wastewater treatment increased efficiency of organic compound removals to greater than 95 percent, [87] but these systems have not been consistently effective in achieving removal of novel, specific, toxic compounds.

The Resource Conservation and Recovery Act (RCRA) of 1976 allows the U.S. Environmental Protection Agency to protect water sources by regulating the disposal of hazardous waste under Subtitle C of the Solid Waste Disposal Act, as amended by the Hazardous and Solid Waste Amendments. Regulations published in May of 1980 (40 Code of Federal

Regulations, parts 261-265) identify hazardous chemicals by definition and by listing of specific wastes, and controls the treatment, storage, transport, and disposal of hazardous waste chemicals. Stricter standards with regard to land disposal of hazardous wastes were promulgated for the protection of surface water and ground water resources. The development of genetically engineered microorganisms may be used in bioengineering systems as a Best Demonstrated Available Technology (BDAT) in the future to meet treatment standards under RCRA [119]; the use of genetically engineered microorganisms is currently under development and has not been applied at field-scale.

A bioengineering system that was eliminated for treatment of hazardous wastes under RCRA Subtitle C is land treatment, which incorporates biological processes in conjunction with physical and chemical processes used in the treatment and ultimate disposal of hazardous waste streams by mixing the wastes with soil for protection of water resources.[129] Land treatment has been replaced by another bioengineering system referred to as a prepared bed system for the treatment of hazardous wastes. A prepared bed consists of a lined (clay and synthetic geo-membrane) system with a leachate collection system to prevent contamination of water resources by containing wastes, soil, and water in the contained prepared bed bioreactor system.[182]

In 1980 the first comprehensive federal law addressing releases of hazardous substances into the environment was enacted, the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), or Superfund. The primary goal of CERCLA was to establish a fund (Superfund) and a mechanism to respond to releases of hazardous substances at abandoned or uncontrolled hazardous waste sites that posed a threat to human health and the environment through contamination of surface water and ground water, air, and soil. Under CERCLA, the National Contingency Plan (NCP) outlined the level of cleanup necessary at a Superfund site. The Superfund Amendments and Reauthorization Act of 1986 (SARA), Section 121, Cleanup

Standards, stipulates rules for selection of remedial actions, provides for review of those actions, describes requirements for the degree of cleanup, and mandates conformance with the NCP whenever possible. Bioremediation of contaminated ground water and soil is considered a bioengineering process under CERCLA that is capable of achieving a permanent cleanup, as encouraged under SARA. However, attainment of regulatory limits with the use of bioremediation is site- and chemical-specific.

The Toxic Substances Control Act (TSCA) regulates the manufacturing of toxic chemicals (chemicals that are linked to cancer, gene mutations, or birth defects), and the disposal of polychlorinated biphenyls (PCBs). TSCA also prohibited the production and distribution of PCBs after July 1979. The U.S. Environmental Protection Agency has interest in regulating biotreatment technologies, including genetically engineered microorganisms, under the TSCA biotechnology program promulgated in 1986 (51 Federal Register 23: 313, June). Laboratory scale as well as field applications of genetic engineering for cleanup of target chemicals in water are largely still in the developmental stages.[95, 156] The use of genetically engineered microorganisms must be evaluated within the context of ecological constraints.[1]

3.2 *Regulatory Acceptance of Bioremediation*

Regulatory acceptance of bioremediation of specific organic compounds in water environments (and especially in aquifers) is sometimes limited by several factors, including[164, 225]: (1) inadequate understanding of the scientific fundamentals of bioremediation, including the biological, chemical, and environmental factors that are required to guarantee the success of the technology; (2) lack of successfully completed and documented field demonstration projects; (3) unpredictable transformation rates leading to uncertain cleanup times, poor process control, and uncertainty in costs; (4) uncertain environmental impacts, due to the formation of harmful transformation products; (5) lack of treatment objectives that are commensurate with the capabilities of bioremediation; (6) presence of multiple contaminants, since bioremediation may

only apply to a few; (7) concern for potential of greater environmental damage if bioremediation fails; and (8) unwillingness to negotiate flexibility in setting cleanup goals that can be accomplished with bioremediation.

3.3 Regulatory Cleanup Levels

At a site with a contaminated aquatic environment that has been designated for cleanup activities under a regulatory program such as CERCLA (better known as Superfund) or the RCRA Corrective Action Program, the degree of cleanup required is usually defined on a site-by-site basis. Three commonly used conservative methods of deciding what is clean include[155]: (1) risk assessments; (2) federal drinking water standards; and (3) analytical detection limits.

Cleanup levels based on risk assessment can be developed specifically for a particular site, with consideration of pathways of human contact and design methods used to prevent human contact.[155] However, there are no standard methods defined for conducting risk assessments, and even when the same method is used, the use of different assumptions will result in different results. Therefore, regulatory agencies often will not use risk assessment to define cleanup levels required; if they are used, because of the variabilities in approaches and assumptions possible, an extended period of discussion among the regulators, consultants, industrial representatives, and the public may result before final decisions are made.

Federal drinking water standards undergo extensive evaluation before acceptance; thus they have a strong technical basis. [155] However, not all compounds of environmental and health significance are covered by federal drinking water regulations. In addition, in contaminated aquifers, organic contaminants are often sorbed to the aquifer solid materials and provide a continuous source of contamination to the ground water. Drinking water standards address only concentrations of compounds in water, but do not address allowable concentrations that can be associated with solid materials.

Analytical detection limits are sometimes used to set cleanup levels.[155] Their use may be

appropriate when the target organic compounds are highly toxic or when numbers determined during a risk assessment are less than the detection limits of the target compounds. However, analytical detection limits are usually continuously improved as analytical technology improves, and the required cleanup levels could be changed over the period of the remedial action.

In some cases, when cleanup levels are set very low, bioremediation may not be effective for meeting the goals. Microorganisms have a minimum level of pollutant substrate required to maintain their metabolic activities. Below those levels, biodegradation of the pollutant may slow or cease. However, if the pollutant is being degraded under cometabolic processes, very low levels may be achievable, for the microorganisms are not using the pollutant as a carbon or energy source.

4.0 BIOENGINEERING TECHNOLOGIES FOR THE ACCOMPLISHMENT OF BIOREMEDIATION

4.1 Use of Bioengineering Technologies for the Removal of Specific Organic Compounds from Contaminated Water

Biological engineering systems have been developed for treatment of different contaminated water sources, including surface and ground waters. Treatment may also be required before the use of a specific water source, as with drinking water, or after use, as with municipal or industrial wastewater. Bioremediation of contaminated surface and waste waters is usually accomplished using a bioengineered contained liquid bioreactor system. In liquid bioreactors, toxic and hazardous pollutants are brought into contact with microorganisms to accelerate the degradation process. For contaminated ground water, the ground water can be extracted from the ground by pumping and subsequently treated in an above-ground reactor

(referred to as pump-and-treat), or treated *in situ* using bioremediation. [11, 44, 155, 171, 183] *In situ* biological treatment of contaminated ground water is usually less costly than pump-and-treat systems using above-ground reactors (either physical, chemical, or biological) but is less easily controlled.[45] A thorough understanding of the subsurface hydrology, geology, and geophysical properties is required in order to engineer a process to manage the subsurface for bioremediation. Pump-and-treat systems, though able to be more directly controlled, are more capital intensive. In addition, even after extensive pump-and-treat operations, often a significant amount of residual contamination may remain in the aquifer sorbed to the solid materials. An overview of bioengineering treatment systems is given in Table 5. *In situ* bioremediation of off-shore oil spills was attempted as part of the cleanup effort of the Exxon Valdez spill off the coast of Alaska during 1989.[104, 162] Commercial fertilizers that were oleophilic in nature (i.e., they tend to adhere to oil) were used to enhance biodegradation. [192] Because degradation takes place at the oil/water interface, these fertilizers are designed to be accessible to and stimulate growth and degradation potential of oil-degrading bacteria. The use of the fertilizers appeared to enhance degradation by two to five times, but some researchers have questioned the statistical significance of the results. In addition, environmentalists have concerns about the toxicity of the oleophilic fertilizers to humans and wildlife.

Because no single unit operation or process can usually treat every contaminant found in a contaminated water source, two or more unit operations may be combined into a treatment train.[45, 185] A treatment train might consist of a mixture of physical (e.g., air stripping, carbon adsorption, ion exchange, and membrane separation), chemical (e.g., precipitation, oxidation/reduction, hydrolysis) and biological processes. Conventional municipal wastewater treatment systems usually consist of a treatment train that incorporates physical and chemical settling processes for solids, chemical processes for the removal of nutrients, and biological processes for the removal of organic compounds. Treatment trains for ground water could

consist of (1) physical removal of non-aqueous phase liquids, followed by *in situ* bioremediation, (2) pump-and-treat, followed by re-injection of the treated water into the aquifer for further treatment *in situ* using bioremediation; or (3) containment of the water compartment using hydraulic barriers created through pumping ground water, and/or physical barriers using bentonite-based walls, in order to create an underground reactor where the water compartment can be biologically treated through biostimulation and/or bioaugmentation.

A difficulty in performing and completing a bioremedial action, such as *in situ* bioremediation of ground water, is that during the project, the concentrations of the target compounds decrease in a non-linear fashion. [155] As the remediation progresses, the rate of decrease in concentration decreases. The required cleanup levels may be set at the asymptotic part of the curve, which may mean the time required for cleanup may be very long, resulting in additional costs, but with little additional treatment occurring during the final stages of cleanup. Also, technologies appropriate for treatment of organic compounds at concentrations present at the beginning of cleanup may be different than technologies appropriate for treatment of concentrations at the end of the remedial action, necessitating a treatment train approach to design. There may be even be a point in a remedial action at which the engineered treatment system could be discontinued, and naturally occurring biodegradation could be used to complete the remedial process to the desired level, with appropriate monitoring and containment activities continuing.

Descriptions of state-of-the-practice and state-of-the-art applications of bioremediation for the treatment of toxic and hazardous organic compounds in bioreactor systems as well as in *in situ systems* are available. Examples of information sources include: (1) proceedings from a conference on on-site and *in situ* bioremediation, [100, 101] sponsored by Battelle Memorial Institute (another conference is planned for 1993); (2) a newsletter, *The Bioremediation Report*, published monthly by COGNIS , Inc., of Santa Clara, CA, which reports on both technical and business developments in bioremediation; (3) proceedings from numerous conferences sponsored every

year by the Hazardous Materials Control Research Institute of Silver Spring, MD; (4) proceedings from conferences sponsored by the Biosystems Technology Development Program of the U.S. Environmental Agency[206]; (5) proceedings of the international conferences on ground water quality and subsurface restoration [151]; (6) reports of the U.S. Environmental Agency program to identify international treatment technologies for hazardous remediation in the United States[152, 154]; and (7) a monthly newsletter, *Bioremediation in the Field*, published by the U.S. Environmental Protection Agency, Cincinnati, OH. The Superfund Innovative Technology Evaluation (SITE) Program of the U.S. Environmental Protection Agency, which was established in 1986, encourages the development and implementation of innovative treatment technologies for hazardous waste site remediation. [207] Bioremedial technologies that are being investigated as part of the SITE Program are described in Table 6.

4.2 Use of Bioengineered Bioreactor Systems

Historically, bioremediation has been used for the treatment of wastewaters; treatment effectiveness has been determined based on the ability of the treatment process to reduce oxygen-demanding materials and nutrients in the waste effluent as well as to reduce the pathogenic nature of the wastes. However, bioremediation now is also being used to remove toxic and hazardous organic compounds from ground and surface waters, including potential drinking water sources, as well as from municipal and industrial waste streams. The use of bioreactors, as compared to uncontained *in situ* systems, may provide the following advantages[192]: (1) greater process management and control; (2) increased contact between microorganism and contaminants; (3) ability to use specific cultures or inoculum more easily; and (3) decreased acclimation times or faster biodegradation rates.

In some cases, bioreactors are designed to remove specific organic contaminants from a specific contaminated water, such as the use of an above-ground bioreactor for treatment of contaminated ground water at a Superfund site. Often, bioreactors at publicly owned treatment

works (POTWs) used for the treatment of municipal wastewater may be utilized for the removal of specific organic compounds. Advantages in the use of POTWs for the treatment of hazardous wastes include[203]: (1) a diverse biomass that can be acclimated to many wastes; (2) dilution of toxic effects of the waste; and (3) availability of nutrients.

Though bioremedial technologies being used for the treatment of toxic and hazardous organic compounds have not changed in any fundamental sense from technologies used in conventional wastewater treatment, their effectiveness for the removal of toxic organic compounds may differ from their effectiveness in the removal of oxygen-demanding substances. Continued research is required to evaluate and to adapt conventional bioremedial treatment processes for the treatment of xenobiotic compounds, which are often present in very low, but still environmentally significant, concentrations in contaminated water sources. [10, 86, 193, 221]

4.2.1 Fate and Transport of Organic Compounds in Bioengineered Bioreactor Systems

Fate and transport mechanisms (i.e., chemical mass balance considerations) that affect hazardous organic constituents in a water or wastewater treatment system include [17]: (Bhattacharya 1992): (1) volatilization; (2) sorption; (3) chemical transformation (abiotic reactions); and (4) biodegradation. The bioengineer should account for these chemical mass balance considerations in order to ensure that the target chemicals are being destroyed and not simply transferred to another environmental compartment. For example, in conventional aerobic processes, volatilization is not controlled. However, if volatilization is expected to be a major pathway of loss of hazardous organic compounds from the system, the use of closed systems may be required (e.g., anaerobic processes). Alternatively, in aerobic processes, the use of fine bubble diffusers and deep tanks increases oxygen transfer efficiency, requires lower air flow rates, and minimizes losses of volatile compounds. Sorption of hazardous organic compounds into biological sludge produced in bioreactors will cause the sludge to be hazardous, and will impose special disposal requirements.

A review of the transport and fate of toxic materials in wastewater facilities was prepared by a group of researchers at Michigan Technological University and Clemson University for the Water Pollution Control Research Foundation. [9] They evaluated present methodology available for determining the fate of a toxic substance in a wastewater treatment facility, focusing on whether it was simply removed from a waste stream and discharged to land or air, or whether the substance was transformed to harmless end products. In a comparison of six comprehensive fate and transport models, using five organic toxic compounds in a hypothetical treatment plant, model simulations showed wide differences in distribution of the toxic compounds among the various fate paths for the different models. As a result of their assessment, they identified the following critical research needs in several major areas:

(1) determination of biokinetic constants and physical properties for pollutants, including:

(a) relationship of sorption of pollutants and their availability for biodegradation, including means to enhance desorption or solubilization in order to promote biodegradation;

(b) factors that control acclimation periods;

(c) rate and extent of biodegradation of pollutants in complex mixtures;

(d) effects of inhibitors on biodegradation;

(e) development and expansion of database of biokinetic rate constants, obtained using standardized protocols, for compounds of regulatory interest and developed for specific treatment technologies where biodegradation represents a significant fate mechanism;

(f) development of predictive methods for determining biokinetic rate constants, such as Quantitative Structure-Activity Relationships (QSARs);

(g) quantitation of biomass population in order to determine what fraction of biomass actively participates in degradation processes;

(h) analysis of the biomass population to determine the extent of sorption of organic compounds on the microbial biomass and residual accumulation in the biomass, which could be mistaken for biodegradation; and

(i) effect of waste matrix (e.g., presence of oils and surfactants) on volatilization of contaminants from wastewater treatment systems;

(2) development of methods for testing and calibration of fate and transport models, including:

(a) development of testing equipment; and

(b) collection of field measurements of mass balances of organic compounds in full-scale treatment facilities;

(3) sensitivity analyses to determine parameters that affect biokinetic rate constants; and

(4) development of a fate and transport model based on a consensus of scientific and engineering opinion.

4.2.2 Types of Bioengineered Bioreactor Systems

Two major types of bioreactors are used for the treatment of hazardous organic constituents in contaminated water [17, 193]: (1) attached-growth; and (2) suspended growth. Both of these types of systems involve the use of naturally occurring immobilized whole cells for the removal of organic wastes from the contaminated water. [65] In attached-growth systems, cells are immobilized in their own polymer matrix onto a surface, such as stone or plastic, in the form of a "biofilm." In suspended-growth systems, cells bind to each other through physical force interactions and in a polymer matrix to form "flocs." Reviews of biofilm formation and kinetics of substrate removal at biofilm/liquid interfaces have been prepared by the research teams of W.G. Characklis [42, 43, 201] and C.S. Criddle?[52]

4.2.2.1 Attached-Growth Bioreactor Systems

Attached-growth bioreactors (sometimes referred to as fixed-film bioreactors) are often used because microorganisms are not wasted from the system, and the SRT is high, which allows for the production of a large biomass volume and the acclimation of the microorganisms to inhibitory compounds in the contaminated water, while minimizing the HRT.[17] In these systems, the biomass is attached to an immobile carrier medium within the bioreactor. Attachment is

accomplished by the secretion of microbial polymers that form a sticky or slimy matrix in which the microorganisms are embedded. [98] The type of media, e.g., plastic or stone, affects the type of microorganisms attached to the media and their metabolic activity. Problems sometime occur when freely suspended microorganisms secrete enzymes that degrade the matrix in which the microorganisms are embedded; this may be a problem especially during start-up. The concentration of suspended microorganisms is controlled by controlling the hydraulic flow, which washes the suspended microorganisms out of the system. Aeration can be provided by diffusion from the atmosphere or by moving air at a countercurrent to the water flow. [158] Attached-growth bioreactors are fairly easy to control, but several problems with their operation have been identified[17]: (1) clogging of the media with biomass; (2) sensitivity to changes in temperature; (3) high costs for capital equipment; and (4) less operating flexibility than the suspended growth systems. Examples of aerobic attached-growth systems include trickling filters, rotating biological contactors, and aerobic fluidized bed bioreactors for wastewater treatment and slow sand filtration for treatment of drinking water sources.

A trickling filter is a bioreactor in which randomly placed solid media provide surface area for biofilm growth. [159] The media often consist of crushed stone or rock, ranging in size from 50 to 100 mm in diameter and with porosities of 40 to 50 percent. The reactor is not actually a "filter," for organic compounds are not removed by physical filtration processes, but by sorption and subsequent biological degradation. Application of wastewater onto the media is accomplished by a rotating distribution arm. The jet action through the nozzles in the arm is usually sufficient to power the rotor. The wastewater is intermittently dosed, with air circulating through the pores between dosing. An underdrain system is used to carry away the treated wastewater and biomass that has sloughed from the media. A high organic loading may result in excessive microbial growth and plugging of the pores, but an increase in hydraulic loading will usually increase sloughing and keep the beds unclogged. Trickling filters are extremely sensitive to temperature

variations, because of the biomass/water/air interfaces, and effluent quality decrease during cooler seasons. Trickling filters have traditionally been important for wastewater treatment because of their simplicity and low operating costs, but requirements for high quality effluent on a consistent basis have made their use less popular.

Rotating biological contactors, used in the treatment of both municipal and industrial wastewaters, consist of a number of corrugated disks mounted on a central shaft, which in turn is mounted lengthwise on a horizontal cylindrical tank, which holds the solution to be treated.[107] The disks are rotated slowly (1 to 2 rpm), with about 40 percent submerged in the bath. During rotation, a film of wastewater is carried on the surface of the disks out of the solution and into the air, where oxygen from the air dissolves into the wastewater. Microorganisms form a biofilm on the disks, where aerobic biodegradation reactions occur. The media must be covered to protect the system from climatic factors, as well as to minimize algal growth. The RBC system is a relatively new process, and experience with full-scale applications is limited.[145] Also the system has a high capital cost and is sensitive to low temperatures. The RBC has been used for growth of white-rot fungi for the degradation of specific organic compounds.[82]

In the aerobic fluidized bed method of contaminated water treatment, sand, with a particle diameter of usually less than 0.3 mm, is used as the support media on which the biofilm develops.[49] The sand particles are suspended in a vertical column by an upward flow of the water that is to be treated. The flow rate is adjusted so that the sand particles are kept in motion but are not swept out of the system with the treated effluent. These beds have a large surface area available for treatment, resulting in effective treatment of organic wastes in a minimum of time and area. This type of system also has an ability to withstand shock loadings of wastes with high levels of biodegradable compounds, i.e., wastes with high BOD levels. However, costs of the process are high, because air or oxygen are required to keep the particles in motion.

A bioengineering process used in the treatment of surface water sources of drinking water

is slow rate sand filtration (SSF).[130] SSF is a bioengineering system that has been successfully used for providing potable water since the nineteenth century. SSF combines physicochemical processes with biological processes for the removal of turbidity,[68, 189] cysts that can cause giardiasis,[102] bacteria,[68, 140] viruses,[140, 141] and dissolved organic chemicals from water.[47, 48] A mat of biological growth, schmutzdecke, composed of algae, bacteria, and other microorganisms including protozoa and rotifers develops primarily on top of a bed of sand approximately 3 feet deep.[144] The filters are operated at low filtration rates, resulting in long retention times.

4.2.2.2 Suspended-Growth Bioreactor Systems

The activated sludge process is the best-known example of the group of treatment systems referred to as suspended-growth systems, in which the biomass is suspended in the liquid phase of the bioreactor.[45] Activated sludge systems have been a standard in the treatment of municipal wastewaters for many years, for they are efficient in the removal of suspended and dissolved organic materials, nutrients, and some trace minerals.[158] However, control of these systems can be fairly complex. The basic system consists of a large basin into which contaminated water is introduced, along with air or oxygen, utilizing diffusion (in which bubbles are produced from submerged porous media such as tubes, plates, or grids) or mechanical aeration devices (such as rotating brushes or surface impellers). The microorganisms involved in degradation are present in the aeration basin as suspended material. The microorganisms are kept in suspension and distributed through the reactor volume by the aeration process. The microorganisms are separated periodically from the water by gravity settling, after which a portion of the settled biomass is returned to the aeration basin, while the remainder is removed as biological sludge for treatment and disposal.

If settling of the microorganisms does not occur, or occurs too slowly, the microorganisms can be washed out of the system, producing a turbid effluent and making the microorganisms unavailable for recycle to the aeration basin.Two kinds of organisms are

involved in a complex and poorly understood process that results in the settling of sludge: floc-forming bacteria and fungi and filamentous bacteria. Floc-formers produce exudates and surface structures that promote the adhesion of microbial cells to each other, forming clumps of hundreds to thousands of cells. Filamentous microorganisms grow in long fiber-like sheaths that cross-link the flocs formed by the floc formers. If too few filaments are present, the flocs are not sufficiently cross-linked (referred to as the development of pin-point flocs) and tend to wash out, producing a turbid effluent. Too many filaments will result in the development of a loose and large floc particle that is of such bulk and "fluffiness" that it will not settle, referred to as a bulking sludge. Though little is known about the mechanisms of floc formation,[76] conditions such as dissolved oxygen, organic loading, nutritional balance (e.g., iron, phosphorus, nitrogen) as well as reactor design (continuous or plug flow), reactor operation (e.g., settling time, mixing, aerator type) and shear stress on the flocs have been shown to affect the development of the filaments. Currently chemically-assisted bioflocculation, using ferric salt, alum, and/or polyelectrolyte coagulant aids, is used during periods of inadequate flocculation. Improved settling of activated sludges is an area that requires continued research. Genetic engineering of microorganisms with desirable floc-forming characteristics has been suggested as a means of improving floc formation. [114]

Waste stabilization lagoons are suspended-growth microbial systems that rely on the symbiotic relationship between autotrophic algae and aerobic heterotrophic bacteria to treat organic wastes.[158] In this process, wastewater is channeled into a basin, in which it is retained for a period of several weeks, compared to a retention time of several hours for wastewaters in other types of aerobic processes.[76] Bacteria metabolize the organic carbon present in the waste, producing new cells and carbon dioxide. The carbon dioxide is used as a carbon source for algae and blue-green bacteria, which grow and produce oxygen, which in turn is then used by the bacteria for metabolism of the wastes. High treatment efficiencies for the removal of nutrients and organic compounds can be achieved, especially in shallow lagoons where aeration and light

penetration allow for the optimization of photosynthetic and bacterial oxidation processes. Specific algae have been shown to be able to metabolize organic compounds that are often recalcitrant in bacterial systems; this degradation is only observed in the presence of light. [37, 38] To enhance aeration, lagoons may be aerated by mechanical agitation or by compressed air diffusion. In these systems, the dominant microorganisms include aerobic heterotrophic bacteria as well as facultative anaerobic heterotrophic bacteria. In deep systems, an aerobic upper layer and an anaerobic lower layer may form. Soluble organic transformation products from anaerobic microbial metabolism may diffuse into the upper layer and be metabolized under aerobic conditions.

4.2.2.3 Anaerobic Attached-Growth and Suspended-Growth Bioreactor Systems

Anaerobic systems, which are used in environments that are depleted of oxygen, sulfates, and nitrates, but utilize CO_2 as the electron acceptor, have been used for about 100 years for the treatment of sewage sludges from wastewater treatment plants. Recently these types of systems have also been utilized in the treatment of wastewaters with high concentrations of organic contaminants. Advantages of anaerobic digestion include [17]: (1) conversion of the organic contaminants almost quantitatively to a high energy fuel, methane; (2) production of less sludges (i.e., new biomass) because anaerobic organisms have a low growth yield coefficient, Y (however, anaerobic organisms are not slow-growers, for their k values (i.e., the maximum rate of substrate use per unit weight of microorganisms), which determine the rate at a waste can be biodegraded, are not low); (3) no energy required for aeration; and (4) efficient treatment of volatile compounds, since anaerobic systems must be closed to prevent the entry of oxygen. Examples of anaerobic processes include anaerobic contact processes, anaerobic filters, anaerobic fluidized bed reactors, and upflow anaerobic sludge blanket systems (Table 7). Anaerobic systems are sometimes built in two-stage configurations, separating the acidification stage (which includes hydrolytic, acidogenic, and acetogenic microorganisms) of the process from the methanogenesis

stage, in order to optimize environmental conditions for the different microbial consortia.

4.2.2.4 Combined Anaerobic/Aerobic Bioreactor Systems

The application of bioreactors is not limited to the use of either aerobic or anaerobic microorganisms, but combined systems, utilizing anaerobic and aerobic processes in sequence, may be used to treat certain waste organic compounds that require anaerobic degradation for initial break-down. Sequential anaerobic/aerobic treatment was used for the successful degradation of hexachlorobenzene, tetrachloroethene, and carbon tetrachloride in a biofilm column reactor system.[215] The first stage of treatment consisted of a methanogenic biofilm column reactor fed acetate as the primary cometabolic substrate. Reductive dechlorination of the target compounds resulted in the formation of mono- and di-chlorinated transformation products. The effluent from the methanogenic biofilm reactor containing these transformation products was fed to an aerobic biofilm reactor seeded with settled sewage. The mono- and di-chlorinated compounds were effectively utilized by the aerobic biofilm.

4.2.3 Economic Considerations in the Use of Bioreactors

Costs of bioreactor systems for the treatment of contaminated water sources are dependent on the following factors[17]:

(1) Cost of primary substrate, if required, for systems utilizing cometabolism;

(2) Cost of nutrients, if required;

(3) Cost of sludge handling (dependent on the amount of sludge generated; may account for 50 percent of the total cost of treatment);

(4) Cost of source of alkalinity for pH control (complicated by cost versus ease and safety of handling);

(5) Cost of pumping;

(6) Relationship between operational and capital costs (i.e, anaerobic bioreactors require large volumes of capacity, but operating costs may be lower compared to aerobic processes that

require aeration and more sludge disposal capacity); and

(7) Treatment of any volatile end or by-products (i.e., costs of treatment of volatile transformation products using activated carbon can be very high).

4.3 In Situ Bioremediation of Ground Water

In situ bioremediation of ground water is being implemented for some contaminated aquifers. Not long ago the subsurface was thought to be sterile, but recent research has shown that the subsurface may contain bacterial populations up to one million organisms per gram of dry soil or aquifer material. [77] Fungi and protozoa have also been identified in the subsurface, but at lower levels.[79] Many of these microorganisms thrive at low levels of organic carbon, but grow poorly or not at all under high nutrient conditions. [78] The bacteria appear to have storage granules, which allow their survival during periods of extended starvation. [219] Most of the bacteria identified are aerobic, but anaerobic bacteria have also been identified. [79]

The most commonly used technique for *in situ* bioremediation is biostimulation of indigenous microorganisms under aerobic conditions by adding nutrients and an oxygen source.[153] However, both *in situ* aerobic and anaerobic bioremedial processes that use primary substrates to encourage cometabolism of target compounds that are susceptible to cometabolic processes are also being investigated, as well the potential of stimulating bioremediation using anaerobic processes by adding alternate electron acceptors such as nitrate. Application of anaerobic processes may be desirable in ground waters because ideal aerobic growth conditions, which includes adequate supplies of nutrients and oxygen, mixing, and a high microbial mass are difficult to maintain in aquifers.[126] Aquifers may also have a high abiotic oxygen demand due to hydrogen sulfide, reduced iron, or other readily oxidizable compounds, making it difficult to maintain an well-oxidized environment. Aerobic biodegradation is usually faster than anaerobic biodegradation, but in an aquifer, the rate of degradation may be limited by mass transfer limitations, such as slow dissolution, dispersion, and/or desorption of the contaminants. Slower

degradation due to anaerobic processes or natural non-stimulated processes may be sufficiently fast for removing contaminants that are sorbed to aquifer materials and are slowly released to the ground water. However, when subsurface conditions do not provide a conducive environment for indigenous microorganisms, or do not allow transport of nutrients, oxygen, or other amendments to the contaminants and microorganisms, treatment in place may not be sufficiently effective or even possible.

The California Biotechnology Action Council has concerns about the possible ecological impacts of added nutrients and other amendments on the functioning of the natural ecosystem, and recommends that analysis of such impacts should be a part of every bioremedial investigation. [192]

4.3.1 Design and Operating Considerations for *In Situ* Bioremediation of Contaminated Aquifers

The most common design of an *in situ* system uses a combination of injection wells (or galleries or trenches for shallow aquifers) and one or more recovery or extraction wells. [153] A typical configuration would be a series of injection wells distributed parallel to the extraction wells. Withdrawal of water faster than it is being reinjected creates a hydraulic gradient that induces ground-water flow to the withdrawal point. This operational technique also results in more effective hydraulic containment of the contaminated ground-water plume and increases the flow of nutrients through the aquifer. Greater depth to ground water at a contaminated site allows greater head at the injection points, which results in greater potential injection rates. Shallower water tables limit the head that can be attained, and are more favorable to the use of injection galleries or trenches.

Recovered ground water may be treated (using an air stripper tower, activated carbon, an oil/water separator, a biological treatment unit, an advanced oxidation unit, or combinations of these treatment units) prior to amendment with nutrients, an oxygen source or alternate electron acceptor, and/or a primary substrate for cometabolism. [153] If the water has been treated in an above-ground bioreactor, pollutant-degrading microorganisms will also be present in the treated

effluent, and will re-injected into the aquifer with the treated water. Alternatively, cultures of microorganisms specifically developed for treatment of the target contaminants may be added to the treated water. After treatment and the addition of amendments, the ground water is re-injected into the contaminated aquifer, where bioremediation of the receiving ground water should be enhanced by the presence of the amended, treated ground water. However, at some sites, the introduced ground water may push away the native ground water containing the contaminants, so that the required mixing between the contaminants and the amendments does not occur. However, if the contaminants are sorbed to the aquifer materials, they will desorb into the introduced water, and contact with the amendments will be accomplished. At some sites, the extracted ground water is not treated in an above-ground reactor, but re-injected after the addition of the required amendments. In this system, as the contaminants are mixed and reinjected with the amendments, desired contact between the contaminants and the amendments is achieved, and the costs of the system are also reduced. In addition, most above-ground treatment reactors involve physical/chemical processes, such as air stripping or carbon adsorption, which only transfer the contaminants to another environmental medium, but do not result in the their destruction , such as would occur when they are re-injected and subsequently biodegraded.

Another system undergoing evaluation for delivering amendments to ground water is subsurface ground-water recirculation, which eliminates the need to pump ground water to the surface for above-ground treatment and addition of amendments. [138] This mixing method utilizes a subsurface sealed recirculation well with an upper and lower screen. A pump is installed in the well to induce flow through the well and through the aquifer. Amendments can be introduced directly into the circulating water through the well.

To utilize *in situ* bioremediation at a specific site, the ground-water flow rate must be sufficient to deliver the required amounts of nutrients, oxygen, and/or other amendments in a reasonable time frame. [153] In addition, the flow paths of the amended ground water should cover the entire area requiring treatment. Recovery wells should be sited to prevent migration outside

the designated treatment zone. To maintain control and containment of the ground water, usually only a portion of the recovered ground water is reinjected, and the other is discharged by an acceptable method. Regulatory permits are usually required for the disposal of the ground water that is not reinjected.

If light non-aqueous phase liquids are floating on the surface of the contaminated ground water, they should be removed using a dual phase pump or skimmer before implementation of bioremediation.[153] Treatment of the unsaturated zone (for example, using *in situ* vapor stripping) will reduce the source of contaminants to the ground water and reduce the time for ground-water cleanup.

4.3.1.1 Addition of Electron Acceptors

When oxygen is injected as the electron acceptor, oxygen requirements are based on stoichiometric relationships (e.g., 3 pounds of oxygen to convert 1 pound of hydrocarbon to carbon dioxide and water).[153] The limit of dissolved oxygen that can be delivered from air is about 8 to 10 ppm, unless injection occurs substantially below the water table. Use of pure oxygen instead of air can increase the amount of oxygen introduced by five times. Sources of oxygen include liquid oxygen, gaseous oxygen, and hydrogen peroxide. On-site generation of oxygen may be accomplished by using zeolite columns to act as a molecular sieve to remove nitrogen from air.[163] Hydrogen peroxide, which decomposes to oxygen and water, is completely soluble in water and may provide levels of oxygen in water 5 to 50 times greater than air injection. Concentrations of hydrogen peroxide are limited to 100 to 1,000 ppm, due to toxicity to bacteria.

An innovative method to increase the delivery of oxygen to aquifers has recently been developed that involves the use of surfactants to create air microbubbles.[146] A microdispersion of very fine air or oxygen bubbles is formed in a surfactant solution using a venturi or spinning disk. Air sparging is also being investigated as a means of enhancing oxygen transfer to aquifers.[27, 28, 133, 147] With air sparging, air is directly injected into a ground-water formation through a well

that contains no water. In addition to increasing oxygen levels in the ground water, air sparging also results in enhanced dissolution of organic chemicals, thus increasing bioavailability. Another advantage of air sparging is that it results in the volatilization of volatile organic contaminants into the unsaturated zone above the water table, where they can be removed from the soil by a soil vapor stripping system.

In systems where methanotrophic cometabolism is being stimulated, methane is added as a primary substrate, in addition to oxygen. [138] Both methane and oxygen are of limited solubility in water. Therefore, added concentrations of these two gases together with other gaseous components such as molecular nitrogen must be below the saturation partial pressure in the aquifer, which may not be much higher than one atmosphere in shallow ground waters.

Anaerobic degradation is biostimulated by adding alternate electron acceptors to the aquifer. Alternate electron acceptors (except for ferric ion) are more water soluble than oxygen. Therefore, lower volumes of amended solutions need to be supplied to the aquifer.[164] Lower biomass yields due to anaerobic growth reduces plugging problems associated with microbial growth, and without additional oxygen, iron precipitation is less of a problem. At this time, nitrate is the only alternate electron acceptor with demonstrated potential for use in large scale *in situ* bioremediation applications. [111, 164] Nitrate has been used as an electron acceptor in field studies of bioremediation of aquifers contaminated with various types of fuels at Traverse City, MI (JP-4 jet fuel spill)[112]; Borden, Ontario (gasoline spill, including benzene, xylenes, and toluene)[16]; Seal Beach, CA (gasoline spill)[165]; and Rhine Valley, Federal Republic of Germany (suspected fuel oil spill).[217]

The use of biostimulated anaerobic processes in aquifers used for drinking water may cause problems with water quality.[24] Under anaerobic conditions, metals such as iron and manganese will become solubilized, which can cause taste, odor, and staining problems. Copper, cadmium, lead and zinc oxides may also become solubilized and enter the distribution system. [69]

Metabolites excreted by the anaerobic microorganisms increase the organic matter content of the water. When the water is disinfected to control pathogens, disinfection by-products, which are regulated by the Safe Drinking Water Act of 1986, can form as the disinfectants react with the organic matter.

4.3.1.2 Addition of Nutrients

Nutrient requirements are based on the mass of organic contaminant to be degraded and can be approximated by a ratio of carbon to nitrogen to phosphorus of 120:10:1. [153] Requirements are adjusted by the following factors: nutrients already present in the contaminated aquifer, nitrogen fixed by indigenous organisms, nutrients recycled from dead bacteria, and sorption of nutrients by aquifer materials. Nutrients commonly used include ammonium chloride and sodium orthophosphate salts. In aquifers high in clay content, the use of sodium salts may reduce the permeability of the aquifer, and potassium salts should be used instead. Tripolyphosphates, when used in a molar ratio equal to or greater than 1:1, solubilize and sequester iron, calcium, and magnesium rather than precipitate these minerals, as may occur when orthophosphates are used, and are recommended for use in certain aquifers.

4.3.1.3 Difficulties Associated with the Addition of Amendments for Biostimulation

The delivery of large quantities of electron donors, electron acceptors, and nutrients to an aquifer can present an engineering challenge, especially when cometabolic processes are being stimulated. For example, in the cometabolic conversion of chlorinated solvents under anaerobic conditions, the mass of primary substrate (electron donor) to mass of chlorinated solvent biotransformed may range from 100:1 to 1000/1. [24] In addition, the high levels of chemicals added are converted to large amounts of end-products, such as methane gas, carbon dioxide, and biomass. These products may adversely affect the bioremedial process in the aquifer, e.g., biomass growth may plug the pore space, reducing the permeability of the aquifer, and interfering with further addition of amendments required for the bioremedial process.

Very high concentrations of contaminants in aquifers, especially in aquifers with lower

permeabilities, will create very high electron acceptor and/or nutrient demands, which may result in excessively long remediation periods and higher costs.[153] Viscous materials, such as heavier fuel oil blends, may prevent the flow of water and the diffusion of nutrients and electron acceptors through a contaminated aquifer, and prevent the implementation of bioremediation. The concentration at which this may occur is site-specific, but is usually above 20,000 mg/kg. Non-degradable contaminants in a contaminated aquifer should also be quantified. If they are above regulatory limits, another technology may be required in addition to bioremediation as part of a treatment train, or another technology should be selected as the remedial option.

Capacities of recovery and injection wells may decrease with time, due to movement of fines, precipitation of minerals, and excessive growth of microorganisms in or in the vicinity of the injection wells.[153] Ground water in a contaminated aquifer is usually in a reduced condition due to utilization of dissolved oxygen by naturally occurring biodegradation, with elevated levels of reduced, soluble iron. With the introduction of recovered, treated ground water to the aquifer, oxygen is added, and iron and other metals, as they become oxidized, may precipitate, which can reduce the permeability of the aquifer and hinder the distribution of the added nutrients, electron acceptors, or other amendments. The addition of nutrients in surges of high concentrations rather than in continuous addition at low concentrations may reduce the tendency of microbial growth in the well bore and in the vicinity of the injection well. Dilute hydrochloric acid may be added to remove mineral deposits and treat excessive microbial growth in the area of the injection wells. High levels of hydrogen peroxide may also be used to treat excessive microbial growth, and is preferred for this purpose, since its use results in the formation of a more flocculent dead microbial mass than the use of acidification, which results in the formation of a slimy mass.

Determination of concentrations of contaminants sorbed to the aquifer solid materials or associated with immiscible phases is extremely important in estimating time required to accomplish the bioremedial action, since these contaminants serve as a continual source to the

ground water through time. [153] Concentrations of contaminants decrease with time through biodegradation, thus enhancing the rate of desorption from the solids or dissolution of an immiscible phase. Therefore, analysis of aquifer solid materials for the presence of contaminants is required in addition to analysis of ground water, in order to estimate the total mass of contaminants requiring remediation. Determination of the total mass of contaminants is difficult to achieve due to heterogeneity in aquifer solid materials as well as heterogeneity in contaminant concentrations associated with the aquifer solid materials or immiscible phases.

Bioremediation is more easily implemented in aquifers with higher permeabilities (i.e., greater than 10^{-3} cm/sec).[153] Nutrients and electron acceptors can be transported more easily to the contamination and there is less sorption of both contaminants and nutrients to the aquifer solid materials, since clay and organic matter content is usually lower in aquifers with higher permeabilities. Heterogeneity in an aquifer formation also complicates understanding of ground water flow direction and rate, and thus control of the remedial action.

4.3.2 Monitoring of *In Situ* Bioremediation

Monitoring wells within the treatment area are used to [153]: (1) determine distribution of nutrients and oxygen; (2) monitor pH and other ground-water chemistry parameters that may impact bioremediation or system operation; (3) monitor ground-water chemistry parameters that are impacted by bioremediation (e.g., removal of electron acceptors, release of waste products such as carbon dioxide or methane) in order to assess the extent of biodegradation occurring; (3) measure ground-water elevations to evaluate ground-water flow; and (4) assess changes in contaminant concentrations to evaluate the extent of biodegradation. An extensive monitoring well network is required to evaluate changes in amount of contaminants present, since dispersion will reduce concentrations of contaminants even if no degradation has occurred. Wells should be located to monitor flows in different directions and at distances that produce changes in water quality, as determined by predicted flow times. Nutrient and electron acceptor distribution can be

adjusted by changing the relative rates of ground-water recovery or reinjection in the wells, or by installing additional wells. If nutrients or contaminants are detected outside the treatment zone, a reduction in the amount of recovered ground water that is being injected may be required.

4.3.3 Application of *In Situ* Bioremediation to Specific Organic Compounds

The properties and biodegradability of the contaminants present at the site will affect the rate and extent of bioremediation. [153] For example, at sites contaminated with petroleum hydrocarbons, lighter, more soluble constituents will biodegrade more rapidly and to lower residual levels than heavier, less soluble constituents that tend to sorb to aquifer solid materials. [7] Monoaromatic compounds such as benzene, toluene, ethylbenzene, and xylenes are more rapidly degraded than two-ring compounds, such as naphthalene, which are more rapidly degraded than three-, four-, and five-ring compounds.[153] Smaller aliphatic compounds are generally degraded more readily than larger compounds, and branched hydrocarbons degrade more slowly than straight chain hydrocarbons. Therefore, gasoline will degrade more rapidly and to a greater extent than heavier products such as No. 6 fuel oil or coal tar. The extent of conversion of gasoline may be limited by the distribution of nutrients and electron acceptors, while the conversion of heavier petroleum hydrocarbons is more likely to be limited by their rate of solubilization , their release from aquifer solid materials, or their rate of degradation.

Non-chlorinated solvents, such as alcohols, ketones, esters, carboxylic acids and esters, are usually readily biodegradable, but may be toxic at high concentrations due to their high solubility in water.[153] Toxicity is in some cases site-specific, since microbial communities have the ability to acclimate to higher concentrations of contaminants.

Chlorinated solvents also have high water solubilities (e.g., 1 g/L), which is several orders of magnitude higher than the drinking water standards of those that are regulated. [153] If chlorinated solvents are present at high concentrations, they may inhibit the biodegradation of other organic wastes present, such as petroleum hydrocarbons. Since chlorinated solvents are

denser than water (referred to as dense non-aqueous phase liquids (DNAPLs)) and have a low tendency to adsorb to soil and aquifer materials, they often penetrate deeply beneath ground water table. Chlorinated solvents, especially the lightly chlorinated, can be degraded under aerobic conditions, but usually require the addition or presence of cometabolites, such as toluene, phenol, propane, ethylene, cresol, ammonia, isoprene, vinyl chloride, or methane[64, 66, 67, 94, 97, 122, 210, 216] Two field pilot-scale studies at the Moffett Naval Air Base have been conducted to evaluate the aerobic degradation of chlorinated solvents using [105, 168, 177, 178]: (1) methane as the primary substrate to stimulate methanotrophs and the production of methane mono-oxygenase to cometabolize the chlorinated solvents; and (2) phenol to stimulate the production of toluene oxygenase by phenol-utilizing bacteria. Ground water extracted from the treatment zone was amended with oxygen and the primary growth substrate and reinjected to stimulate the growth of indigenous microorganisms. Above-ground treatment was not used; the chlorinated solvents extracted with the ground water were re-injected into the aquifer. Conclusions from the studies included: (1) stimulation of indigenous methanotrophs and phenol-utilizers was accomplished with the addition of the primary substrates and oxygen; (2) rates and extent of transformation were compound-specific, with removal rates ranging from 20 to 95 percent ; (3) the rates of transformation were limited by the rates of desorption of the target compounds from the aquifer solids; and (4) the cometabolic transformations were competitively inhibited by the primary substrates, resulting in the reduction of the transformation rate; and (5) results correlated with laboratory microcosm studies, which mimicked the conditions of the field tests.

Under anaerobic conditions, chlorinated solvents, especially those that are highly chlorinated, can also be degraded, but appropriate environmental conditions and microorganisms must be present for these reactions to occur. Also, some of the transformation products may be more hazardous than the parent compounds. The transformation of carbon tetrachloride by acetate-utilizing denitrifying bacteria was investigated at the field site at the

Moffett Naval Air Base under the mildly reducing conditions of denitrification.[176] Ground water was extracted from the site and amended with acetate and nitrate. Conclusions from the study included: (1) stimulation of indigenous acetate-utilizers was accomplished with the addition of acetate and nitrogen; (2) carbon tetrachloride was transformed through reductive dechlorination reactions, with an average removal rate of 95 percent; and (3) chloroform was observed to be an undesirable transformation product, as was observed in laboratory microcosm studies. Concentrations of carbon tetrachloride below typical health-based standards of 5 to 10 ug/L were not achieved in this field study. A laboratory study, however, has shown that carbon tetrachloride can be transformed to CO_2, with residual concentrations below regulatory limits [53], so if these anaerobic processes can be optimized, they can meet relevant regulatory endpoints. [24]

The potential for degradation of chlorinated solvents using sequential anaerobic/aerobic processes has been investigated in a laboratory-scale aquifer simulator containing contaminated aquifer materials and ground water. [57] During the anaerobic portion of the study, a recirculation flow of glucose and nutrients was used to maintain methanogenic conditions, during which time tetrachloroethene and trichloroethene were degraded to dichloroethene. Oxygen was then introduced, and the oxidation of dichloroethene was accomplished by methanotrophic bacteria.

Until more full-scale experience is available, *in situ* bioremediation should perhaps be limited to use at sites that contain more readily degradable contaminants, that are relatively simple hydrogeologically, and that have a well-defined point source of contamination with only one or two contaminants (e.g., spills, leaking underground storage tanks, and simple manufacturing sites) rather than multiple and undefined sources of contamination. [138] Also, complete information should be made public concerning the types of living microorganisms and other amendments used at the site. [103] The use of bioremediation in this conservative manner will build public confidence as well as a reliable database of information. For example, bioremediation could be a potential effective remedial technique for an aquifer contaminated with

vinyl chloride, which is a known human carcinogen with very low regulatory limits.[138] Vinyl chloride is difficult and expensive to treat in conventional pump-and-treat systems, because it does not sorb well to activated carbon or other sorbent materials. However, it can be degraded *in situ* as a primary substrate as well as through cometabolism by methanotrophic bacteria, with only two kilograms of methane required per kilogram of vinyl chloride degraded.

4.3.4 Natural Bioremediation

Recently, recommendations have been made to utilize "natural (or passive) bioremediation" of aquifers in conjunction with and to supplement conventional remediation techniques to cleanup certain contaminated aquifers. [22] For example, pump-and-treat operations could be used to reduce concentrations of contaminants within an aquifer to some defined level, at which time pump-and-treat operations would be terminated, and the natural biodegradation processes of indigenous microorganisms occurring in the aquifer would be used to complete the cleanup. At this time there are no full-scale demonstrations of natural bioremediation used specifically to cleanup a site, but work has been performed investigating those processes that control the natural biodegradation of dissolved contaminant plumes. [22, 46, 84, 85, 223]

In natural bioremediation of a contaminated ground water plume, dissolved contaminants are degraded as they are transported down-gradient within the aquifer. [22] At the point where the contaminants enter the ground water from a source in the unsaturated zone, indigenous aerobic microorganisms will degrade the contaminants until the oxygen is used up. Because the solubility of oxygen in water is relatively low, only a small amount of the contaminants will be degraded. The contaminants that are not degraded will be carried down-gradient in the plume of anaerobic, contaminated water. As the plume migrates, dispersion will result in the mixing of the anaerobic contaminated water with clean oxygenated water at the plume fringes. After a period of acclimation, aerobic degrading bacteria will develop in the aquifer solids of this fringe area. As the oxygenated water mixes with contaminated water, the attached bacteria will utilize both the

contaminants and the oxygen, thus preventing the further spread of the contaminated plume. As dissolved contaminants disperse outward, they come in contact with the oxygenated ground water and are biodegraded. If this process is allowed to continue indefinitely, the dissolved contaminated plume will reach a quasi-steady state condition, where the long term rate of dissolution of contaminants from the source area is equal to the rate of biodegradation. In the core of the plume, conditions may become highly reducing, and anaerobic degradation processes may occur. Any organic contaminant degraded through anaerobic processes will reduce the oxygen demand on the aquifer, and result in more oxygen being available for those compounds that can only be degraded aerobically. The extent of aerobic biodegradation will be controlled by the amount of contamination released, the rate of oxygen transfer into the subsurface, the background oxygen content of the aquifer, and the environmental conditions present in the aquifer. In addition, heterogeneous conditions in the aquifer will prevent mixing and will allow the plume to migrate quickly down-gradient.

To utilize natural bioremediation for the cleanup of an aquifer, the source of contamination should be removed, followed by careful monitoring of system performance. A monitoring system typically includes[22]: (1) interior wells to monitor the plume distribution and indicator parameters; and (2) guardian wells at the outside edge of the area of contamination to detect potential off-site migration and to determine if additional remedial actions are required. Typical indicator parameters measured in the interior wells include [22]: (1) individual target contaminants, to determine extent of bioremediation; (2) dissolved oxygen, to determine if biodegradation is occurring, as well as to delineate the contaminant plume; (3) nitrate and dissolved iron, to assess the extent of anaerobic degradation; (4) redox potential, to assess the overall oxidation-reduction status of the aquifer; (5) carbon dioxide and pH, to evaluate the extent of microbial respiration and to determine if conditions are suitable for bioremediation; and (8) total organic carbon, to evaluate the extent of the contaminated plume, to monitor the production of organic transformation products, and to evaluate the extent of biodegradation. The guardian

wells are usually used for regulatory purposes and are primarily monitored for target contaminants.

An assessment of the distance a plume will migrate before contaminants are biodegraded is also required for the implementation of natural bioremediation. [22] This assessment requires an estimation of the rate of migration and the rate of biodegradation of the contaminants. The rate of contaminant migration can be estimated by measuring the hydraulic gradient and the permeability of the aquifer. Estimation of the rate of biodegradation within the aquifer is much more difficult. Modelling tools have been developed to predict the rate of natural biodegradation. An example of such a model is BIOPLUME II , which was developed to simulate hydrocarbon degradation. [166] This model incorporates advection, dispersion, oxygen-limited biodegradation, and first order decay in a two-dimensional aquifer. BIOPLUME II does not simulate dissolution of hydrocarbons nor the anaerobic degradation of hydrocarbons, which may result in an under-estimate of biodegradation.

The use of natural bioremediation at this time is limited by lack of acceptance of the approach by regulators, environmental groups, and the public. [22] These groups are concerned with the lack of control of the process as well as uncertainties whether public health and the environment will be protected without a definitive assurance of success. Though costs of operating such a system should be low, these low costs may be offset by substantial costs required to adequately characterize the site as well as costs to monitor the progression of natural bioremediation.

4.3.5 Regulatory Considerations for the Use of *In Situ* Bioremediation

Regulatory targeted endpoint contaminant concentration levels vary significantly at specific sites. These levels can be State-mandated levels, Federal-mandated levels, or risk-based levels. State regulations are sometimes the most difficult to meet, since they are often set at detection limit or at background levels. The use of non-specific parameters, such as total petroleum hydrocarbon

(TPH) levels, as remediation goals may cause misleading conclusions about system performance.[153] TPH analyses often measure components that are not of interest, such as asphalt particles, do not measure the most volatile compounds, and can yield highly variable results. Some states (e.g., New Jersey) require that final nutrient concentration levels be at or below background levels at the end of the remedial effort, which requires continuous, careful monitoring of nutrient levels used during the remedial process.

With regards to specific contaminants, the most difficult regulatory endpoint to meet is usually for benzene, for as a carcinogen, the MCLs for benzene are usually an order of magnitude lower than for other light hydrocarbon constituents. [153] If the benzene endpoint is met, levels for other components are usually met also. For heavier petroleum hydrocarbons, TPH is a typical target analysis. However, during treatment, there will probably be residuals of slowly degraded compounds with low water solubilities in the aquifer. TPH analyses do not distinguish which hydrocarbon constituents have not been treated. Also, compounds that are not of environmental concern also contribute to TPH values and hinder interpretation of the effectiveness of the bioremediation system.

Polyaromatic hydrocarbons are often difficult to treat to regulatory levels in contaminated ground water.[153] As suspected carcinogens, their MCLS are set very low. Their degradation rate is usually slow, they are associated with aquifer solid materials, and are only slowly released to the ground water. At low concentrations, they may not be able to support an active degrading population of organisms. Degradation is usually enhanced if other more degradable compounds are present, which support an active degrading population of organisms.

4.3.6 Economic Considerations for the Use of *In Situ* Bioremediation

Costs of implementing *in situ* natural bioremediation under aerobic conditions using indigenous microorganisms are dependent on the following factors [153]:

(1) Mass of contaminants - affects amount of nutrients and electron acceptors required,

time required to achieve acceptable remediation, as well as capital expenditure for wells, pumps, and above-ground treatment reactors;

(2) Volume of contaminated aquifer - affects number of injection and recovery points required and the time required to achieve acceptable remediation;

(3) Permeability of aquifer materials - affects number of injection and recovery points required and the time required to achieve acceptable remediation;

(4) System design - results in higher capital expenditure costs at sites with more injection and recovery wells, but may reduce the operating and maintenance costs by reducing the total time of remediation;

(5) Selection of electron acceptor - impacts costs, with more expensive sources, such as hydrogen peroxide, increasing monthly operating costs but decreasing overall operating costs by reducing the period of operation of the remedial activity;

(6) Final remediation levels - results in higher costs with more stringent remediation goals, especially for sites with containing contaminants that are recalcitrant to biodegradation and that are poorly soluble;

(7) Depth to ground water - results in higher costs for installation of wells at lower depths, but costs can be balanced by greater flows of injected water at depth, due to increased pressure head, resulting in shorter times required for remediation;

(8) Monitoring requirements - affects costs considerably, depending on the number of wells to be monitored, frequency of monitoring required, and number and type of parameters requiring measurement;

(9) Contaminant properties - affects the amount of contaminants that can be recovered in the ground water, thus affecting the residual concentrations remaining in the aquifer that must be bioremediated *in situ* and the costs of the bioremedial process. Increased concentrations in the recovered ground water may result in increased costs for above-ground treatment. Important properties include solubility and tendency to be associated with aquifer solid materials (i.e,

partition coefficients); and

(10) Site location - affects the cost of labor, with remote sites having higher travel and housing costs, especially at sites that have highly automated operations, where the presence of monitoring personnel are only required periodically.

Examples of typical system costs are given in Table 8.

5.0 Current State of Knowledge Regarding Applications and Limitations for Bioengineering

In July, 1991, a workshop on "Utilizing Bioremediation Strategies: Difficulties and Limitations" was held, organized by Rutgers University and sponsored by the U.S. Environmental Protection Agency, New Jersey Department of Environmental Protection, U.S. Navy, National Institute of Environmental Health Sciences, and Environment Canada. A guidance document, based on the discussions conducted at the workshop and compiled by the Interdisciplinary Bioremediation Working Group of Rutgers University, was developed to advance the field of bioremediation by facilitating communication and decision-making about choices of bioremediation treatments and approaches to implementing such treatments, within the contexts of known limitations of the technology![113]

The following problems with the utilization of bioremediation were identified, as well as some proposed solutions, approaches, and factors for consideration [113]:

(1) *Bioremediation assessment and implementation requires more integrated efforts across disciplines* -

Continued basic science and engineering research is required to develop the full potential of bioremediation of contaminated water sources. Interdisciplinary research representing microbial biochemistry, genetics, ecology, environmental microbiology, hydrogeology, and

chemical and environmental bioprocess engineering must be encouraged from project inception to completion by project managers with the expertise to communicate with and coordinate scientist and engineers from different disciplines.[173] The U.S. National Research Council has identified several reasons that inhibit collaborative research, including [209]: (1) differences in conceptual approach, resulting in language and communication barriers; (2) lack of career incentives and rewards due to institutional and organizational constraints; and (3) differences in formal training and orientation among practitioners of different disciplines.

The use of models (conceptual, mathematical, and physical) and knowledge-based decision-making systems are powerful tools for integrating and focusing information from separate disciplines. Their development should be a high priority for research. Information flow from laboratory to field and back should be iterative, involving professionals from all appropriate disciplines.

(2) *Initial site characterizations can be inadequate to evaluate or employ bioremediation as a treatment alternative* -

The use of scientifically and statistically valid sampling plans during initial site characterization should be employed to determine environmental heterogeneity and to measure relevant physical and chemical parameters that affect biological activity, such as pH, salinity, temperature, available electron acceptors, and presence and chemical redox state of metals (especially iron for contaminated ground-water sites). Site contamination should be well-characterized, including concentrations and distribution. For ground-water contamination, hydrogeological properties and parameters should be determined, including hydraulic conductivity, direction of flow, water table fluctuations, recharge area, and type of aquifer (confined, unconfined).

Appropriate microbiological tests should be conducted to evaluate microbial activity and toxicity of a site to critical microbial populations. Control testing should be conducted (e.g., for contaminated ground water sites, an adjacent background area should be tested). Microbial

testing could include respiration, $^{14}CO_2$ evolution or ATP analysis.

The development of a knowledge-based decision-making system for site characterization would ensure that information was collected in a thorough and efficient manner.

(3) *Standard methods for the performance of treatability study protocols and methods as well as criteria for biotreatability assessments should be developed* -

The goal of a specific treatability study should be well-defined with regards to whether the goal is to obtain basic data concerning biotreatability or whether it is to simulate all or part of the bioremedial process. In the performance of a treatability study, appropriate controls should be incorporated into the study design, including abiotic, killed, and endogenous treatments. There is often variability in degradation kinetics for compounds that exhibit resistance to degradation (Baillod et al.); therefore sufficient replication of testing should be included for valid statistical analysis; non-parametric statistics should be utilized if parametric statistics are not appropriate. Actual field materials should be used in treatability studies, as well as conditions reflecting existing and attainable field conditions.

The use of a mass balance approach for the determination of the fate and transport of target organic compounds is mandatory, including analyses of mineralization, transformation, volatilization, and sorption processes. Appropriate toxicity testing in addition to chemical analyses should be used to evaluate treatment effectiveness.

Rates of biodegradation should be determined to develop estimations of time required for cleanup of a specific contaminated water. Also, probable limiting factors to biodegradation should be considered, and engineering responses to those limitations should be developed.

(4) *Factors limiting degradation rates in bioremediation should be adequately identified and addressed* -

Physicochemical conditions limiting to biodegradation, including factors such as temperature, pH, salinity, electron acceptors, redox potential, nutrients and toxic substances, should be identified. Engineering responses to these limiting conditions should be developed, since

one of the most important barriers to the use of bioremediation is the lack of ability to control and predict the rate and extent of bioremediation at field scale due to the influence of environmental conditions.

Engineering responses may encompass the amelioration of problem conditions (e.g., biostimulation) or the use of appropriate strains of microorganisms resistant to the adverse conditions (i.e., bioaugmentation). Specific approaches include: (1) temperature limitations overcome by the use of contained bioreactors rather than *in situ* treatment; (2) bioavailability of poorly soluble or sorbed organic constituents improved with the use of surface active agents, either added to the system or produced *in situ* by microorganisms (3) transport of water, electron acceptors, nutrients, co-substrates, and introduced microorganisms is ground-water systems by improved control of pumping; (4) microbial biodegradative potential increased by the introduction of degrading microorganisms capable of degrading the target compound(s) after demonstration of efficacy in well-designed experiments incorporating appropriate control treatments; (5) presence of appropriate electron acceptors increased by the addition of, either singly or in combination, oxygen, hydrogen peroxide, nitrates, sulfates, or organic substrates; (6) oxygen transport limitations in contaminated ground water ameliorated by use of above-ground bioreactors; (7) degradation rates enhanced by the use of systems that maintain microorganisms in an active state (e.g., use of immobilized cells); (8) cometabolism promoted by the addition of primary organic substrates; and (9) reductive degradation reactions encouraged with the establishment of appropriate system redox conditions;

Problems due to toxicity may be addressed by dilution, pH controls, treatment for metals (e.g., immobilization, volatilization, chelation), the use of sequential treatments in a treatment train approach to reduce toxicity, or the utilization of microbial strains resistant to the toxic substances. However, more research is required to develop an understanding of limiting conditions, especially as conditions interact to affect biodegradative potential.

(5) *During selection of treatment options for a specific contaminated water, the full range of*

options should be considered to ensure that bioremediation is not prematurely removed from consideration -

The feasibility of using bioremediation for a specific contaminated water source should be incorporated in the decision making process. The type of bioremedial process and well as possible treatment train combinations with physical/chemical processes should be investigated as part of a feasibility study. A knowledge-based decision-making system consisting of decision trees that incorporate relevant site characteristics that affect the selection of an appropriate remedial option or combination of options should be developed.

(6) *Additional information and enhanced modeling principles should be developed to improve scale up from laboratory scale reactors or microcosms to full-scale field systems.*

Laboratory, pilot and field experiments should be linked in an iterative process in order to develop rational scale-up criteria. Both stochastic and deterministic models should be used to identify limiting mechanisms and critical parameters. Inputs to models used should be site-specific, including data concerning limiting conditions. The validation of models should be conducted using pilot scale information. To define operational parameters, best case/worst case scenarios should be used in the modeling efforts.

(7) *Techniques for monitoring field performance, utilizing mass balance concepts require continued development -*

A chemical mass balance approach to evaluating transport and behavior of target contaminants as well as monitoring their concentrations should be used. Protocols for preparing sample plans that include sampling the solid, liquid, and gaseous phases of the system, as appropriate, and that are based on sound scientific and statistical practices, should be developed. Key measurements may include: (1) contaminants; (2) added substrates, nutrients, or electron acceptors; (3) transformation products; (4) toxicity; (5) non-degradable tracers; and (6) microbial populations (specifically contaminant-degraders). Samples should be collected periodically through the bioremedial process to monitor changes in the measured parameters.

(8) *An accessible, thorough, and well-documented database on bioremediation should be developed* -

Field experiments utilizing quantitative measures of treatment effectiveness should be conducted to provide a database for process design. Treatment plans and results for specific bioremedial actions should be reviewed by an external expert review panel. Results of quantitative field experiments and process designs for bioremediation should be published in peer-reviewed journals. Results of case studies should be included as part of remedial technology databases, such as the ATTIC, VISITT[208] and Pesticide Treatability [204] databases of the U.S. Environmental Protection Agency.

The Bioremediation Action Committee of the U.S Environmental Protection Agency also sponsored a workshop on bioremediation in 1991. [205] The purpose of the workshop was to identify high priority topics for research to further advance bioremedial technologies. Four major areas of research were identified [205]: (1) to determine factors governing the availability of pollutants for bioremediation and devise ways to increase their availability for biodegradation; constraints on availability include sorption/desorption processes, pollutants present in non-aqueous phase liquids, matrix effects, weathering and aging of pollutants, and immobilization and solubilization processes; (2) to improve the design of processes, including management of limiting factors, development of effective monitoring processes, development of multi-stage processes and treatment trains, and determination of factors that affect the success of bioaugmentation; (3) to overcome problems associated with scale-up from simple laboratory systems to field operations, and (4) to develop innovative and novel bioremediation approaches and processes, including toxicity reduction approaches, cometabolic, anaerobic/aerobic multi-stage, and microaerophilic processes, and methods to control transformation pathways. Results of research addressing the above items should greatly expand the scope of use of bioremediation for the cleanup of contaminated waters in surface and subsurface environments.

6.0 Conclusion

With regard to the use of bioengineering for cleanup of contaminated water, challenges for the multi-disciplinary area of bioengineering include focusing scientists and engineers beyond historical and traditional general classes of pollutants to novel, individual, specific, and often toxic chemicals that are recalcitrant with regard to biological transformations. Understanding of processes that relate biological activity to physical and chemical characteristics of the environment are needed in order to design systems to control and enhance bioremediation for water cleanup.

7.0 Bibliography

1. Alexander, M.: Ecological constraints on genetic engineering. *Hazardous Waste Treatment by Genetically Engineered or Adapted Organisms.* Hazardous Materials Control Research Institute, Silver Spring, MD; 1984.

2. Alexander, M.: Why microbial predators and parasites do not eliminate their prey and hosts? *Annual Reviews in Microbiology* 35:113-133; 1981.

3. Alexander, M.: Research needs in bioremediation. *Environmental Science and Technology* 25 (12): 1972-1973; 1991.

4. Altenschmidt, U.; & Fuchs, G.: Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: Indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. *Archives of Microbiology* 156: 152-158; 1991.

5. Ames, B.N.; McCann, J.; & Yamasaki, E.: Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Research* 31: 347-364; 1975.

6. Armenante, P.M.; Lewandowski, G.A.; & Pak, D.: Reactor designs employing a white rot fungus for hazardous waste treatment. Lewandowski, G., Armenante, P., and Baltzis, B., editors: *Biotechnology Applications in Hazardous Waste Treatment.* Engineering Foundation, New York, NY; 1989.

7. Atlas, R.M.: *Microbiology: Fundamentals and Applications.* 2nd Edition. Macmillan Publishing Co., New York, NY; 1988.

8. Bailey, J. E.; & Ollis, D.F.: *Biochemical Engineering Fundamentals, Second Edition.* McGraw-Hill, New York, NY; 1986.

9. Baillod, C.R.; Crittenden, J.C.; Mihelcic, J.R.; & Rogers, T.N.: *Critical Evaluation of the State of Technologies for Predicting the Transport and Fate of Toxic Compounds in Wastewater Facilities.* WPCF Research Foundation Project 90-1, Water Pollution Control Federation Research Foundation, Alexandria, VA; 1991.

10. Baltzis, B.C.; Lewandowski, G.A.; Chang, S.H.; Ko, Y.F.: Fill-and-draw reactor dynamics in biological treatment of hazardous wastes. Lewandowski, G., Armenante, P., and Baltzis, B., editors: *Biotechnology Applications in Hazardous Waste Treatment.* Engineering Foundation, New York, NY; 1989.

11. Barcelona, M.; Wehrman, A.; Keely, J.F.; & Pettyjohn, W.A.: *Contamination of Ground Water: Prevention, Assessment, Restoration.* Pollution Technology Review No. 184, Noyes Data Corporation, Park Ridge, NJ; 1990.

12. Barth, E.F.; & Bunch, R.L.: *Biodegradation and Treatability of Specific Pollutants.* EPA-600/9-79-034, Municipal Environmental Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH; 1979.

13. Basheer, S.; Kut, O.M.; Prenosil, J.E.; & Bourne, J.R: Kinetics of enzymatic degradation of cyanide. *Biotechnology and Bioengineering* 39(6): 629-634; 1992.

14. Benefield, L.D.; Judkins, J.F.; & Weand, D.L.: *Process Chemistry for Water and Wastewater Treatment.* Prentice-Hall, Inc., Englewood Cliffs, NJ; 1982.

15. Berry, D.R.; & Senior, E.: Applications of molecular biology. Sidwick, J.M. & Holdom, R.S., editors: *Biotechnology of Waste Treatment and Exploitation*, Ellis Horwood Limited, Chichester, England; 1987.

16. Berry-Spark, K.L.; Barker, J.F.; MacQuarrie, K.T.; Major, D.; Mayfield, C.I.; & Sudicky, E.E.: *The Behavior of Soluble Petroleum Product Derived Hydrocarbons in Ground Water, Phase III.* PACE Report No. 88-2, Petroleum Association for Conservation of the Canadian Environment, Ottawa, Ontario, Canada; 1988.

17. Bhattacharya, S.K.; How to engineer biological processes that neutralize hazardous wastes. *Remediation: The Journal of Environmental Cleanup Costs, Technologies & Techniques* 2(2):199-210; 1992

18. Bhattacharya, S.K., & Parkin, G.F.: Modeling toxicity kinetics in complete-mix anaerobic systems. *Proceedings, National Conference on Environmental Engineering.* American Society of Civil Engineers, Vancouver, British Columbia; 1988.

19. Bhattacharya, S.K., and Parkin, G.F.: The effect of ammonia on methane fermentation processes. *Journal Water Pollution Control Federation* 61: 55-59; 1989.

20. Blackburn, J.W.: Is there an "uncertainty principle" in microbial waste treatment? Huntley, M.E., editor: *Biotreatment of Agricultural Wastewater.* CRC Press, Boca Raton, FL; 1989.

21. Blackburn, J.W.; Jain, R.K.; & Sayler, G.S.: The molecular microbial ecology of a naphthalene degrading genotype in activated sludge. *Environmental Science & Technology* 21: 884-890; 1987.

22. Borden, R.C.: Natural bioremediation of hydrocarbon contaminated ground water. *Ground-Water Cleanup through Bioremediation.* Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; In press.

23. Borden, R.C.; Bedient, P.B.; Lee, M.D.; Ward, C.H.; & Wilson, J.T.: Transport of dissolved hydrocarbons influenced by reaeration and oxygen limited biodegradation. 2. Field application. *Water Resources Research* 22(1): 1983-1990; 1986.

24. Bouwer, E.J.: Bioremediation of chlorinated solvents using alternate electron acceptors. *Ground-Water Cleanup through Bioremediation.* Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; In press.

25. Bouwer, E.J.; & McCarty, P.L.: Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. *Applied and Environmental Microbiology* 45(4): 1286-1294; 1983.

26. Brown, A.D.: Microbial water stress. *Bacteriological Reviews* 40: 803-846; 1976.

27. Brown, R.A.; Herman, C.; & Henry, E.: The use of aeration in environmental clean-ups. *Proceedings, Haztech International Pittsburgh Waste Conference.* Pittsburgh, PA; 1991.

28. Brown, R.: Treatment of petroleum hydrocarbons in ground water by air sparging. *Ground-Water Cleanup through Bioremediation.* Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; In press.

29. Brown, V.R.; & Knapp, J.S.: The effect of withdrawal of morpholine from the influent and its reinstatement on the performance and microbial ecology of a model activated sludge plant treating a morpholine-containing influent. *Journal of Applied Bacteriology* 69: 43-53; 1990.

30. Bulger, P.R.; Kehew, A.E.; & Nelson, R.A.: Dissimilatory nitrate reduction in a waste-water contaminated aquifer. *Ground Water* 27(5): 664-671; 1990.

31. Bulich, A.A.: Microtox - a bacterial toxicity test with several environmental applications. Liu, D., and Dutka, B.J., editors: *Toxicity Screening Procedures Using Bacterial Systems.* Marcel Dekker, Inc., New York, NY; 1984.

32. Bulich, A.A.: Use of luminescent bacteria for determining toxicity in aquatic environments. Markings, L.L., and Kimerle, R.A., editors: *Aquatic Toxicology.* American Society for Testing and Materials, Philadelphia, PA; 1979.

33. Bumpus, J.A.; Fernando, T.; Jurek, M.; Mileski, G.J.; & Aust, S.D.: Biological treatment of hazardous wastes by *Phanerochaete chrysosporium.* Lewandowski, G., Armenante, P., and Baltzis, B., editors: *Biotechnology Applications in Hazardous Waste Treatment.* Engineering Foundation, New York, NY; 1989.

34. Caldwell, S.R.; & Raushel, F.M.: Detoxification of organophosphate pesticides using an immobilized phosphotriesterase from *Pseudomonas diminuta. Biotechnology and Bioengineering* 37: 103-109; 1991.

35. Cardinal, L.J.; & Stenstrom, M.K.: Enhanced biodegradation of polycyclic hydrocarbons in the activated sludge process. *Journal Water Pollution Control Federation* 63(7): 950-957; 1991.

36. Castro, C.E.; & Belser, N.O.: Biodehalogenation. Reductive dehalogenation of the biocides ethylene dibromide, 1,2-dibromo-3-chloropropane, and 2,3-dibromobutane in soil. *Environmental Science and Technology* 2: 779-783; 1968.

37. Cerniglia, C.E.; Gibson, D.T.; &Van Baalen, C.: Oxidation of naphthalene by cyanobacteria and microalgae. *Journal of General Microbiology* 116: 495- 500; 1980.

38. Cerniglia, C.E.; Van Baalen, C ; & Gibson, D.T.:Oxidation of biphenyl by the cyanobacterium, *Oscillatoria* sp., strain JMC. *Archives of Microbiology* 125: 203-207; 1980.

39. Chakrabarty, A.M., editor: *Biodegradation and Detoxification of Environmental Pollutants.* CRC Press, Inc., Boca Raton, FL; 1982.

40. Chakrabarty, A.M.: Molecular mechanisms in the biodegradation of environmental pollutants. *ASM News* 44: 687; 1978.

41. Chakrabarty, A.M.: Needs and strategies in the application of genetic engineering to clean up synthetic environmental pollutants. Alexander, M., editor: *Microbial Technologies to Overcome Environmental Problems of Persistent Pollutants.* United Nations Environment Programme,

Nairobi, Kenya; 1987.

42. Characklis, W.G.; & Cooksey, K.E.: Biofilms and microbial fouling. *Advances in Applied Microbiology* 29:93-138; 1983.

43. Characklis, W.G.; Trulear, M.G.; Bryers, J.D.; & Zelver, N.: Dynamics of biofilm processes: methods. *Water Research* 16:1207-1216.

44. Charbeneau, R.J.; Bedient, P.B.; & Loehr, R.C.: *Groundwater Remediation.* Technomic Publishing Co., Lancaster, PA; 1992.

45. Cheremisinoff, P.N.; & Goessmann, G.: Comparing groundwater remediation options. *Remediation: The Journal of Environmental Cleanup Costs, Technologies & Techniques* 2(2):153-169; 1992.

46. Chiang, C.Y.; Salanitro, J.P.; Chai, E.Y.; Colthart, J.D.; & Klein, C.L.: Aerobic biodegradation of benzene, toluene, and xylene in a sandy aquifer - data analysis and computer modeling. *Ground Water* 27(6): 823-834; 1989.

47. Collins, M.R.; Eighmy, T.T.; Fenstermacher, J.M., Jr.; & S.K. Spanos, S.K.: *Modifications to the Slow Sand Filtration Process for Improved Removals of Trihalomethane Precursors.* American Water Works Foundation Research Report, AWWA Research Foundation, Denver, CO; 1989.

48. Collins, M.R.; Eighmy, T.T.; Fenstermacher, J.M., Jr.; & S.K. Spanos, S.K.: Removing natural organic matter by conventional slow sand filtration. *Journal of the American Water Works Association* 84(5): 80-90; 1992.

49. Cooper, P.F.: The use of biological fluidised beds for the treatment of domestic and industrial waste waters. *Chemical Engineer* 371:373-376; 1981.

50. Corbitt, R.A.: *Standard Handbook of Environmental Engineering.* McGraw-Hill, New York, NY; 1989.

51. Crawford, R.L.; & O'Reilly, K.T.: Bacterial decontamination of agricultural wastewaters. Huntley, M.E., editor: *Biotreatment of Agricultural Wastewater.* CRC Press, Boca Raton, FL; 1989.

52. Criddle, C.S.; Alvarez, L.M.; & McCarty, P.L.: 1991. Microbial processes in porous media. Baer, J. and Corapcioglu, Y., editors: *Transport Processes in Porous Media.* NATO ASI Series, Kluwer Academic Publishers, Boston, MA; 1991.

53. Criddle, C.S.; DeWitt, J.T.; Grbic-Galic, D.; & McCarty, P.L.: Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrification conditions. *Applied and Environmental Microbiology* 56(11): 3240-3246; 1990.

54. Day, S.M.: Federal regulations: How they impact research and commercialization of biological treatment. Sayler, G.S., Fox, R., and Blackburn, J.W., editors: *Environmental Biotechnology for Waste Treatment.* Plenum Press, New York, NY; 1991.

55. DeWeerd, K.A.; Concannon, F.; & Suflita, J.M.: Relationship between hydrogen

consumption, dehalogenation, and the reduction of sulfur oxyanions by *Desulfomonile tiedjei*. *Applied and Environmental Microbiology* 57(7): 1929-1934; 1991.

56. DiStefano, T.D., Gossett, J.M.; & Zinder, S.H.: Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. *Applied and Environmental Microbiology* 57(8): 2287-2292; 1991.

57. Dooley-Danna, M.; Fogel, S.; & Findlay, M.: The sequential anaerobic/aerobic biodegradation of chlorinated ethenes in an aquifer simulator. *Proceedings, International Symposium on Processes Governing the Movement and Fate of Contaminants in the Subsurface Environment*. Stanford University, Palo Alto, CA; July 23-26, 1989.

58. Dragun, J.: *The Soil Chemistry of Hazardous Materials*. Hazardous Materials Control Research Institute, Silver Spring, MD; 1988.

59. Dutka, B.J.; & Bitton, G., editors: *Toxicity Testing Using Microorganisms*. CRC Press, Inc., Boca Raton, FL; 1986.

60. Dwyer, D.; Krumme, M.; Boyd, S.; & Tiedje, J.: Kinetics of phenol biodegradation by an immobilized methanogenic consortium. *Applied and Environmental Microbiology* 52: 345-351; 1986.

61. Edwards, E.; Wills, L.E.; Grbic-Galic, D.; & Reinhard, M.: Anaerobic degradation of toluene and xylene - evidence for sulfate as the terminal electron acceptor. Hinchee, R.E., and Olfenbuttel, R.F., editors: *In Situ Bioreclamation, Applications and Investigations for Hydrocarbon and Contaminated Site Remediation*. Butterworth-Heinemann, Boston, MA; 1991.

62. Edwards, E.; Wills, L.E.; Reinhard, M.; & Grbic-Galic, D.: Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. *Applied and Environmental Microbiology* 58: 794-800; 1992.

63. Eveleigh, D.E.: A perspective on the development of regulations for release of genetically engineered microbes. Kamely, D., Chakrabarty, A., & Omenn, G.S., editors: *Biotechnology and Biodegradation*, Advances in Applied Biotechnology Series, Volume 4, Gulf Publishing Co., Houston, TX; 1989.

64. Ewers, J.; Clemens, W.; & Knackmuss, H.J.: Biodegradation of chloroethenes using isoprene as co-substrate. *Proceedings of International Symposium: Environmental Biotechnology*. European Federation of Biotechnology, Ostende, Belgium; April 22-25, 1991.

65. Flint, K.P.: Immobilized whole-cell biocatalysts. Sidwick, J.M. & Holdom, R.S., editors: *Biotechnology of Waste Treatment and Exploitation*, Ellis Horwood Limited, Chichester, England; 1987.

66. Fogel, M.M.; Taddeo, A.R.; & Fogel, S.: Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. *Applied and Environmental Microbiology* 51: 720-724; 1986.

67. Folsom, B.R.; Chapman, P.J.; & Pritchard, P.H.: Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: Kinetics and interactions between substrates. *Applied and Environmental Microbiology* 56: 1279-1285; 1990.

68. Foreman, G.P.: *Slow Rate Filtration With and Without Clinoptilolite: A Comparison of Water Quality and Filtration Economics.* Master's Thesis, Utah State University, Logan, UT; 1985.

69. Francis, A.J.; & Dodge, C.J.: Anaerobic microbial dissolution of transition and heavy metal oxides. *Applied and Environmental Microbiology* 54(4): 1009-1014; 1988.

70. Fox, J.L.: Confronting doubtful oil clean-up data. *Bio/technology* 9: 14; 1991.

71. Fox, J.L.: EPA seeks high profile for bioremediation. *Bio/technology* 8: 283; 1990.

72. Freedman, D.L.; & Gossett, J.M.: Biodegradation of dichloromethane in a fixed film reactor under methanogenic conditions. Hinchee, R.E., and Olfenbuttel, R.F., editors: *On-Site Bioremediation Processes for Xenobiotic and Hydrocarbon Treatment.* Butterworth-Heinemann, Boston, MA; 1991.

73. Fujita, M.; Ike, M.; & Hashimoto, S.: Feasibility of wastewater treatment using genetically engineered microorganisms. *Water Research* 25(8): 979-984; 1991.

74. Fulthorpe, R.R.; & Wyndham, R.C.: Involvement of a chlorobenzoate-catabolic transposon, Tn5271 in community adaptation to chlorobiphenyl, chloroaniline, and 2,4-dichlorophenoxyacetic acid in a freshwater ecosystem. *Applied and Environmental Microbiology* 58(1): 314-325; 1992.

75. Fulthorpe, R.R.; & Wyndham, R.C.: Survival and activity of a 3-chlorobenzoate-catabolic genotype in a natural system. *Applied and Environmental Microbiology* 55: 1584-1590; 1989.

76. Gaudy, A.F.; & Gaudy, E.T.: *Microbiology for Environmental Scientists and Engineers.* McGraw-Hill Book Co., New York, NY; 1980.

77. Ghiorse, W.C.; & Balkwill, D.L.: Enumeration and morphological characterization of bacteria indigenous to subsurface environments. *Developments in Industrial Microbiology* 24: 213-224; 1983.

78. Ghiorse, W.C.; & Balkwill, D.L.: Microbial characterization of subsurface environments. Ward, C.H., Gieger, W., and McCarty, P.L., editors: *Ground Water Quality.* John Wiley and Sons, New York, NY; 1985.

79. Ghiorse, W.C.; & Wilson, J.T.: Microbial ecology of the subsurface. *Advances in Applied Microbiology* 33: 107-172; 1988.

80. Gibson, D.T., editor: *Microbial Degradation of Organic Compounds.* Marcel Dekker, New York, NY; 1985.

81. Glaser, J.A.: Hazardous waste degradation by fungi. Kamely, D., Chakrabarty, A., & Omenn, G.S., editors: *Biotechnology and Biodegradation.* Advances in Applied Biotechnology Series, Volume 4, Gulf Publishing Co., Houston, TX; 1989.

82. Glaser, J.A.; Tabak, H.M.; Opatken, E.J.; Joyce, T.W.; Chang, H.; Stroehofer, S.; & Hummel, C.: Use of a white-rot fungus in a rotating biological contactor. *Bioremediation of Hazardous Wastes.* EPA/600/9-90/044, Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC; 1990.

83. Godsy, E.M.; & Grbic-Galic, D.: Biodegradation pathways for benzothiophene in methanogenic microcosms. *U.S. Geological Survey Toxic Substances Hydrology Program: Proceedings of the Technical Meeting*. USGS Water-Resources Investigations Report 88-4220, Phoenix, AZ; September 26-30, 1988.

84. Godsy, E.M.; Goerlitz, D.F.; & Ehrlich, G.G.: Methanogenesis of phenolic compounds by a bacterial consortium from a contaminated aquifer in St. Louis Park, Minnesota. *Bulletin of Environmental Contamination and Toxicology* 30: 261-268; 1983.

85. Goerlitz, D.F.; Troutman, D.E.; Godsy, E.M.; & Franks, B.J.: Migration of wood preserving chemicals in contaminated ground water in a sand aquifer at Pensacola, Florida. *Environmental Science and Technology* 19(10) 955-961; 1985.

86. Grady, C.P.L.: The enhancement of microbial activity through bioreactor design. Lewandowski, G., Armenante, P., and Baltzis, B., editors: *Biotechnology Applications in Hazardous Waste Treatment*. Engineering Foundation, New York, NY; 1989.

87. Grady, C.P.L., Jr.; & Lim, H.C.: *Biological Wastewater Treatment: Theory and Applications*. Marcel Dekker, Inc., New York, NY; 1980.

88. Grbic-Galic, D.: Methanogenic transformation of aromatic hydrocarbons and phenols in ground-water aquifers. *Geomicrobiology Journal* 8:167-200; 1990.

89. Grbic-Galic, D.: Microbial degradation of homocyclic and heterocyclic aromatic hydrocarbons under anaerobic conditions. *Developments in Industrial Microbiology* 30:237-253; 1989.

90. Grbic-Galic, D.; & Vogel, T.M.: Transformation of toluene and benzene by mixed methanogenic cultures. *Applied and Environmental Microbiology* 53: 254-260; 1987.

91. Gude, H.: Grazing by protozoa as selection factor for activated sludge bacteria. *Microbial Ecology* 5:225-237; 1979.

92. Hambrick, G.A., III; DeLaune, R.D.; & Patrick, W.H.: Effect of estuarine sediment pH and oxidation-reduction potential on microbial hydrocarbon degradation. *Applied and Environmental Microbiology* 40: 365-369; 1980.

93. Harrison, L.A.: Immobilized enzymes and their applications. Sidwick, J.M. & Holdom, R.S., editors: *Biotechnology of Waste Treatment and Exploitation*, Ellis Horwood Limited, Chichester, England; 1987.

94. Hartsmans, S.; & de Bont, J.A.M.: Aerobic vinyl chloride metabolism in *Mycobacterium aurum* L1. *Applied and Environmental Microbiology* 58: 1220-1226; 1992.

95. Hazardous Materials Control Research Institute: *Hazardous Waste Treatment by Genetically Engineered or Adapted Organisms*. Hazardous Materials Control Research Institute, Silver Spring, MD; 1988.

96. Hedgement, G.D.: The evolution of metabolic pathways in bacteria. Gunsalus, I.C., editor: *Degradation of Synthetic Organic Molecules in the Biosphere*. National Academy of Sciences,

Washington, DC; 1972.

97. Henry, S.M.: *Transformation of Trichloroethylene by Methanotrophs from a Groundwater Aquifer.* Ph.D. Dissertation, Stanford University, Palo Alto, CA; 1991.

98. Henze, M: Process intensification. Sidwick, J.M. & Holdom, R.S., editors: *Biotechnology of Waste Treatment and Exploitation.* Ellis Horwood Limited, Chichester, England; 1987.

99. Heron, G.; & Christensen, T.: Degradation of the herbicide mecoprop in an aerobic aquifer determined by laboratory bench studies. *Chemosphere* 24(5): 547-557; 1992.

100. Hinchee, R.E.; & Olfenbuttel, R.F., editors: *In Situ Bioreclamation, Applications and Investigations for Hydrocarbon and Contaminated Site Remediation.* Butterworth-Heinemann, Boston, MA; 1991.

101. Hinchee, R.E.; & Olfenbuttel, R.F., editors: *On-Site Bioremediation Processes for Xenobiotic and Hydrocarbon Treatment.* Butterworth-Heinemann, Boston, MA; 1991.

102. Hirschi, S.D.: *Biological Aspects of Particle Removal in Slow Rate Sand Filtration.* Master's Thesis, Utah State University, Logan, UT; 1989.

103. Hirschorn, J.: Superfund strategies and technologies: A role for biotechnology. Omenn, G.S., editor: *Environmental Biotechnology: Reducing Risks from Environmental Chemicals through Biotechnology.* Plenum Press, New York; 1988.

104. Hodgson, B.: Alaska's big spill: Can the wilderness heal? *National Geographic*, 177(1): 5-43; 1990.

105. Hopkins, G.D.; Semprini, L.; & McCarty, P.: Evaluation of enhanced in situ aerobic biodegradation of trichloroethylene, and *cis*- and *trans*-1,2-dichloroethylene by phenol utilizing bacteria. *Symposium on Bioremediation of Hazardous Wastes.* Biosystems Technology Development Program, Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC; May 5-6, 1992.

106. Horvath, R.S.: Microbial co-metabolism and the degradation of organic compounds in nature. *Biotechnology Reviews* 36: 146-155; 1972.

107. Howard, H.K.: Biodegradation of aqueous hazardous waste leachates in a pilot-scale rotating biological contactor. Freeman, H.M., and Sferra, P.R., editors: *Biological Processes: Innovative Hazardous Waste Treatment Technology Series, Volume 3.* Technomic Publishing Co., Lancaster, PA; 1991.

108. Howard, P.H.: *Biodegradation Database.* Lewis Publishers, Boca Raton, FL; 1992.

109. Howard, P.H.; & Meylan, W.M.: *Biodegradation Probability Program (Database).* Lewis Publishers, Boca Raton, FL; 1992.

110. Howard, P.H.; Jarvis, W.F.; Meylan, W.M.; & Michalenko, E.M.: *Handbook of Environmental Degradation Rates.* Lewis Publishers, Boca Raton, FL; 1992.

111. Hutchins, S.R.; Downs, W.C.; Wilson, J.T., Smith, G.B.; Kovacs, D.A.; Fine, D.D.

Douglass, R.H.; & Hendrix, D.J.: Effect of nitrate addition on biorestoration of fuel-contaminated aquifer: Field demonstration. *Ground Water* 29(4): 571-580; 1991.

112. Hutchins, S.R.; & Wilson, J.T.: Laboratory and field studies on BTEX biodegradation in a fuel-contaminated aquifer under denitrifying conditions. Hinchee, R.E., and Olfenbuttel, R.F., editors: *In Situ Bioreclamation, Applications and Investigations for Hydrocarbon and Contaminated Site Remediation.* Butterworth-Heinemann, Boston, MA; 1991.

113. Interdisciplinary Bioremediation Working Group: *Utilizing Bioremediation Technologies: Difficulties and Approaches, Report of A National Workshop, July 12-14, 1991.* Center for Agricultural Molecular Biology, Rutgers, The State University of New York, New Brunswick, NJ; 1991.

114. Johnston, J.B.; & Robinson, S.G.: *Genetic Engineering and New Pollution Control Technologies.* Noyes Publications, Park Ridge, NJ; 1984.

115. Keck, J.; Sims, R.C.; Coover, M.; Park, K.; & Symons, B.: Evidence for cooxidation of polynuclear aromatic hydrocarbons in soil. *Water Research* 23: 1467-1476; 1989.

116. Klein, J.; & Kluge, M.: Immobilization of microbial cells in polyurethane matrices. *Biotechnology Letters* 3:65-71; 1981.

117. Klibanov, A.M.; & Morris, E.D.: Horseradish peroxidase for the removal of carcinogenic aromatic amines from water. *Enzyme and Microbial Technology* 3:119-122; 1981.

118. Knackmuss, H-J.: Potential for genetic engineering and other novel technologies to enhance destruction of toxic chemicals. Alexander, M., editor: *Microbial Technologies to Overcome Environmental Problems of Persistent Pollutants.* United Nations Environment Programme, Nairobi, Kenya; 1987.

119. Korwek, E.L.: Federal regulation of hazardous waste treatment by genetically engineered or adapted microorganisms. *Biotreatment: The Use of Microorganisms in the Treatment of Hazardous Materials and Hazardous Wastes, Proceedings of the Second National Conference.* Hazardous Materials Control Research Institute, Silver Spring, MD; 1989.

120. Kuhn, E.P.; & Suflita, J.M.: Dehalogenation of pesticides by anaerobic microorganisms in soils and ground water - a review. Sawhney, B.L., & Brown, K., editors: *Reactions and Movement of Organic Chemicals in Soils.* Special Publication No. 22, Soil Science Society of America, Inc., Madison, WI; 1989.

121. Lajoie, C.A.; Chen, S.Y.; Oh, K.C.,; & Strom, P.F: Development and use of field application vectors to express nonadaptive foreign genes in competitive environments. *Applied and Environmental Microbiology* 58(2): 655-663; 1992.

122. Lanzarone, N.A.; & McCarty, P.L.: Column studies on methanotrophic degradation of trichloroethene and 1,2-dichloroethane. *Ground Water* 28: 910-919; 1990.

123. Leavitt, M.: Simultaneous soil and ground-water bioremediation. *The Hazardous Waste Consultant* 10(5): 4.22-4.23; 1992.

124. LeChevallier, M.W.: Becker, W.C.; Schorr, P.; & Lee, R.G.: 1992. Evaluating the

performance of biologically active rapid filters. *Journal of the American Water Works Association* 84(5): 136-146; 1992.

125. Leisinger, T., editor: Reviews - microbial degradation of environmental pollutants. *Experientia* 39(11):1181; 1983.

126. Lee, M.D.; Thomas, J.M; Borden, R.C.; Bedient, P.B.; & Wilson, J.T.: Biorestoration of aquifers contaminated with organic compounds. *CRC Critical Reviews in Environmental Control* 18: 29-89; 1988.

127. Liu, D.; & Dutka, B.J., editors: *Toxicity Screening Procedures Using Bacterial Systems.* Marcel Dekker, Inc., New York, NY; 1984.

128. Loehr, R.: *Treatability Potential for EPA Listed Hazardous Chemicals in Soil.* EPA/600/2-89/011, Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; 1989.

129. Loehr, R.C.; & Malina., J.F., editors: *Land Treatment - A Hazardous Waste Management Alternative.* Water Resources Symposium Number 13, Center for Research in Water Resources, University of Texas at Austin, University of Texas Press, Austin, TX; 1986.

130. Logsdon, G.S., editor: *Slow Sand Filtration.* American Society of Civil Engineers, New York, NY; 1991.

131. Lovley, D.R.; & Lonergan, D.J.: Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organisms, GS-15. *Applied and Environmental Microbiology* 56: 1858-1864; 1990.

132. Manem, J.A.; & B.E. Rittmann: Removal of trace pollutants in a biological filter. *Journal of the American Water Works Association* 84(5): 152-157; 1992.

133. Marley, M.C.; Walsh, M.T.; & Nangeroni, P.E.: Case study on the application of air sparging as a complimentary technology to vapor extraction at a gasoline spill site in Rhode Island. *Proceedings, HMC Great Lakes 90.* Hazardous Materials Control Research Institute, Silver Spring, MD; 1990.

134. Maron, D.M.; & Ames, B.N.: Revised methods for the Salmonella mutagenicity test. *Mutation Research* 113: 173-215; 1983.

135. McCann, J.R.; Choi, R.; Yamasaki, E.; & Ames, B.N.: Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. *Proceedings of the National Academy of Sciences* 72: 5135-5139; 1975.

136. McCarty, P.L.: Application of biological transformations in ground water. *Proceedings, 2nd International Conference on Ground Water Quality Research.* National Center for Ground Water Research, Tulsa, OK; 1984.

137. McCarty, P.L.: Energetics and bacterial growth. Faust, J., and Hunter, J.V., editors: *Organic Compounds in Aquatic Environments.* Marcel Dekker, Inc., New York, NY; 1971.

138. McCarty, P.L.; & Semprini, L.: Ground-water treatment for chlorinated solvents. *Ground-*

Water Cleanup through Bioremediation. Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; In press.

139. McCarty, P.L.; Rittmann, B.E.; & Bouwer, E.J.: Microbiological processes affecting chemical transformations in ground water. Bitton, G., and Gerba, C.P., editors: *Groundwater Pollution Microbiology.* Wiley-Interscience, New York, NY; 1984.

140. McConnell, L.K.: *Evaluation of the Slow Rate Sand Filtration Process for Treatment of Drinking Water Containing Viruses and Bacteria.* Master's Thesis, Utah State University, Logan, UT; 1984.

141. McConnell, L.K.; Sims, R.C.; & Barnett, B.B.: Reovirus removal and inactivation by slow rate sand filtration. *Applied and Environmental Microbiology* 48(4): 818-825; 1984.

142. McFarland, M.J.; & Sims, R.C.: Thermodynamic framework for evaluating PAH degradation in the subsurface. *Ground Water* 29(6): 885-896; 1991.

143. McFarland, M.J.; Sims, R.C.; & Blackburn, J.W.: Use of treatability studies in developing remediation strategies for contaminated soils. Sayler, G.S., Fox, R., & Blackburn, J.W., editors: *Environmental Biotechnology for Waste Treatment.* Plenum Press, New York, NY; 1991.

144. McNair, D.R.; Sims, R.C.; Sorensen, D.L.; & Hulbert, M.: Schmutzdecke characterization of clinoptilolite-amended slow sand filtration. *Journal of the American Water Works Association* 79(12): 1987.

145. Metcalf and Eddy, Inc.: *Wastewater Engineering: Treatment, Disposal, and Reuse.* McGraw-Hill, New York, NY, 1979.

146. Michelsen, D.L.; Wallis, D.A.; & Sebba, F.: In situ biological oxidation of hazardous organics. *Environmental Progress* 3(2): 103-107; 1984.

147. Middleton, A.C.; & Hiller, D.H.: In situ aeration of ground water - a technology overview. *Proceedings, Conference on Prevention and Treatment of Soil and Ground Water Contamination in the Petroleum Refining and Distribution Industry.* Montreal, Quebec, Canada; 1990.

148. Mouchet, P.: From conventional to biological removal of iron and manganese in France. *Journal of the American Water Works Association* 84(5): 158-167; 1992.

149. Munnecke, D.M.: Detoxification of pesticides using soluble or immobilised enzymes. *Process Biochemistry* 13(2): 14-16, 31; 1978.

150. Nakamoto, S.; & Machida, N.: Phenol removal from aqueous solutions by peroxidase-catalyzed reaction using additives. *Water Research* 26(1): 49-54; 1992.

151. National Center For Ground Water Research: *Subsurface Restoration Conference. Proceedings of the Third International Conference on Ground Water Quality Research.* Rice University, Houston, TX; June 21024, 1992.

152. NATO/CCMS Pilot Study Program: *Demonstration of Remedial Action Technologies for Contaminated Land and Groundwater. Proceedings, Third International Conference.* U.S. Environmental Protection Agency, Cincinnati, OH; November 6-9, 1989.

153. Norris, R.D.: In-situ bioremediation of soils and ground water contaminated with petroleum hydrocarbons. *Ground-Water Cleanup through Bioremediation.* Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; In press.

154. Nunno, T.; Hyman, J.; Spawn, P. Healy, J.; Spears, C.; & Brown, M.: *Assessment of International Technologies for Superfund Applications - Technology Identification and Selection.* EPA/600/2-89/017, Risk Reduction Engineering Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH; 1989.

155. Nyer, E.K.: *Practical Techniques for Groundwater and Soil Remediation.* Lewis Publishers, Chelsea, MI; 1992.

156. Omenn, G.S.; & Hollaender, A.: 1984. *Genetic Control of Environmental Pollutants.* Basic Life Sciences, Volume 28, Plenum Press, New York, NY; 1984.

157. Palmer, D.T.; Linkfield, T.G.; Robinson, J.B., Genthner, B.R.S.; & Pierce, G.E.: *Determination and Enhancement of Anaerobic Dehalogenation: Degradation of Chlorinated Organics in Aqueous Systems.* EPA/600/2-88/054, U.S. Environmental Protection Agency, Cincinnati, OH; 1989.

158. Pearson, S.: Deciding on a treatment alternative. Huntley, M.E., editor: *Biotreatment of Agricultural Wastewater.* CRC Press, Boca Raton, FL; 1989.

159. Peavy, H.S.; Rowe, D.R.; & Tchobanoglous, G.: *Environmental Engineering.* McGraw-Hill, New York, NY; 1985.

160. Perry, J.J.: Microbial cooxidation involving hydrocarbons. *Microbiological Reviews* 43:59-72; 1972.

161. Phelps, T.J.; Niedzielski, J.J.; Malachowsky, K.J., Schram, R.M.; Herbes. S.E.; & White, D.C: Biodegradation of mixed-organic wastes by microbial consortia in continuous-recycle expanded-bed reactors. *Environmental Science and Technology* 25(8): 1461-1465; 1991.

162. Pritchard, P.H.; & Costa, C.F.: EPA's Alaska oil spill bioremediation project. *Environmental Science and Technology* 25(3): 372-379; 1991

163. Prosen, B.J.; Korreck, W.M.; & Armstrong, J.M.: Design and preliminary performance results of a full-scale bioremediation system utilizing an on-site oxygen generator system. Hinchee, R.E., and Olfenbuttel, R.F., editors: *In Situ Bioreclamation, Applications and Investigations for Hydrocarbon and Contaminated Site Remediation.* Butterworth-Heinemann, Boston, MA; 1991.

164. Reinhard, M.: In-situ bioremediation technologies for petroleum derived hydrocarbons based on alternate electron acceptors (other than molecular oxygen). *Ground-Water Cleanup through Bioremediation.* Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; In press.

165. Reinhard, M.; Wills, L.E.; Ball, H.A.; Harmon, T.; Phipps, D.W.; Ridgeway, H.F.; & Eisman, M.P.: A field experiment for the anaerobic biotransformation of aromatic hydrocarbon compounds at Seal Beach, California. Hinchee, R.E., and Olfenbuttel, R.F., editors: *In Situ*

Bioreclamation, Applications and Investigations for Hydrocarbon and Contaminated Site Remediation. Butterworth-Heinemann, Boston, MA; 1991.

166. Rifai, H.S.; Bedient, P.B.; Haasbeek, J.F.; & Borden, R.C.: *BIOPLUME II: Computer Model of Two-Dimensional Contaminant Transport under the Influence of Oxygen Limited Biodegradation in Ground Water, User's Manual - Version 1.0.* EPA/600/S8-88/093, U.S. Environmental Protection Agency, Washington, DC; 1989.

167. Rittmann, B.E.: Needs and strategies for genetic control: Municipal wastes. Omenn, G.S., and Hollaender, A., editors: *Genetic Control of Environmental Pollutants.* Plenum Press, New York, NY; 1984.

168. Roberts, P.V.; Hopkins, G.D.; Mackay, D.M.; & Semprini, L.: A field evaluation of in-situ biodegradation of chlorinated ethenes: Part 1. Methodology and field site characterization. *Ground Water* 28: 591-604; 1990.

169. Rochkind-Dubinsky, M.L.; Sayler, G.S.; & Blackburn, J.W.: *Microbiological Decomposition of Chlorinated Organic Compounds.* Marcel Dekker, New York, NY; 1987.

170. Rubio, M.A.; & Wilderer, P.A.: Process strategies to enhance transfer of plasmid coded degradative properties for chlorinated hydrocarbons in sequencing batch reactors. Lewandowski, G., Armenante, P. and Baltzis, B., editors: *Biotechnology Applications in Hazardous Waste Treatment.* Engineering Foundation, New York, NY; 1989.

171. Sabatini, D.A.; & Knox, R.C.: *Transport and Remediation of Subsurface Contaminants.* ACS Symposium Series 491, American Chemical Society, Washington, DC; 1992.

172. Salkinoja-Salonen, M.; Middeldorp, P.; Briglia, M.; Valo, R.; Haggblom, M.; & McBain, A.: Cleanup of old industrial sites. Kamely, D., Chakrabarty, A., & Omenn, G.S., editors: *Biotechnology and Biodegradation.* Advances in Applied Biotechnology Series, Volume 4, Gulf Publishing Co., Houston, TX; 1989.

173. Sayler, G.S.; & Blackburn, J.W.: Modern biological methods: The role of biotechnology. Huntley, M.E, editor.: *Biotreatment of Agricultural Wastewater.* CRC Press, Boca Raton, FL; 1989.

174. Sayler, G.S.; Kong, H-L.; & Shields, M.S.: Plasmid-mediated biodegradative fate of monohalogenated biphenyls in facultatively anaerobic sediments. Omenn, G.S., and A. Hollaender, A., editors: *Genetic Control of Environmental Pollutants.* Basic Life Sciences, Volume 28, Plenum Press, New York, NY; 1984.

175. Semprini, L.; & McCarty, P.L.: Comparison between model simulations and field results for in-situ bioremediation of chlorinated aliphatics: Part 1. Biostimulation of methanotrophic bacteria. *Ground Water* 29(3): 365-374; 1991.

176. Semprini, L.; Hopkins, G.D.; Janssen, D.B.; Lang, M.; Roberts, P.V.; & McCarty, P.L.: *In-Situ Biotransformation of Carbon Tetrachloride under Anoxic Conditions.* EPA/2-90/060, Robert S, Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; 1991.

177. Semprini, L.; Hopkins, G.D.; Roberts, P.V.; Grbic-Galic, D.; & McCarty, P.L.: A field

evaluation of in-situ biodegradation of chlorinated ethenes: Part 3. Studies of competitive inhibition. *Ground Water* 29(2): 239-250; 1991.

178. Semprini, L.; Roberts, P.V.; Hopkins, G.D., & McCarty, P.L.: A field evaluation of in-situ biodegradation of chlorinated ethenes: Part 2. Results of biostimulation and biotransformation experiments. *Ground Water* 28(5): 715-727; 1990.

179. Sewell, G.W.; & Gibson, S.A.: Stimulation of the reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by the addition of toluene. *Environmental Science and Technology* 25(5): 982-984; 1991.

180. Shaw, J.J.; Dane, F.; Geiger, D.; & Kloepper, J.W.: Use of bioluminescence for detection of genetically engineered microorganisms released into the environment. *Applied and Environmental Microbiology* 58(1): 267-273; 1992.

181. Siahpush, A.R.; Lin, J.-E.; & Wang, H.Y.: Effect of adsorbents on degradation of toxic organic compounds by coimmobilized systems. *Biotechnology and Bioengineering* 39(6): 619-628; 1992.

182. Sims, J.L., Sims, R.C.; & Matthews, J.E.: *Bioremediation of Contaminated Surface Soils*. EPA/600/9-89/073, Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada OK; 1989.

183. Sims, J.L.; Suflita, J.M.; & Russell, H.H.: *In-Situ Bioremediation of Contaminated Ground Water*. EPA/540/S-92-003, Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada OK; 1992.

184. Sims, J.L.; Suflita, J.M.; & Russell, H.H.: *Reductive Dehalogenation of Organic Contaminants in Soils and Ground Water*. EPA/540/4-90/054, Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; 1991.

185. Sims, R.C.: Soil remediation techniques at uncontrolled hazardous waste sites: A critical review. *Journal of the Air & Waste Management Association* 40(5): 704-732.

186. Sims, R.C.; Sims, J.L.; & Dupont, R.R.: Human health effects assays. *Journal Water Pollution Control Federation* 60: 1093-1196; 1988.

187. Sixt, H.; & Sahm, H.: Biomethanation. Sidwick, J.M. & Holdom, R.S., editors: *Biotechnology of Waste Treatment and Exploitation*, Ellis Horwood Limited, Chichester, England; 1987.

188. Slater, J.H.: Genetic interactions in microbial communities. Klug, M.J., and Reddy, C.A., editors: *Current Perspectives in Microbial Ecology*. American Society of Microbiology, Washington, DC; 1984.

189. Slezak, L.A.; & Sims, R.C.: The application and effectiveness of slow sand filtration in the United States. *Journal of the American Water Works Association* 76(12): 38-43; 1984.

190. Smith, G.B.; & Tiedje, J.M.: Isolation and characterization of a nitrite reductase gene and its use as probe for denitrifying bacteria. *Applied and Environmental Microbiology* 58(1): 376-384; 1992.

191. Somerville, H.; Mason, J.; & Ruffell, R.: Benzene degradation by bacterial cells immobilized in polyacrylamide gel. *European Journal of Applied Microbiology* 4: 75- 85; 1977.

192. Stabinsky, D.: *The Overselling of Bioremediation: A Primer For Policy Makers and Activists*. California Biotechnology Action Council, Sacramento, CA; 1991.

193. Stensel, H.D.; & Strand, S.E.: Reactor configurations in hazardous waste treatment. Lewandowski, G., Armenante, P., and Baltzis, B., editors: *Biotechnology Applications in Hazardous Waste Treatment*. Engineering Foundation, New York, NY; 1989.

194. Sterritt, R.M.; & Lester, J.N.: *Microbiology for Environmental and Public Health Engineers*. E. & F.N. Spon, New York, NY; 1988.

195. Stormo, K.E.; & Crawford, R.L.: Preparation of encapsulated microbial cells for environmental applications. *Applied and Environmental Microbiology* 58(2): 727-730; 1992.

196. Suflita, J.M.; & Sewell, G.W.: *Anaerobic Biotransformation of Contaminants in the Subsurface*. EPA/600/M-90/024, Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; 1991.

197. Suflita, J.M.; Gibson, S.A.; & Beeman, R.E.: Anaerobic biotransformation of pollutant chemicals in aquifers. *Journal of Industrial Microbiology* 3:179-194; 1988.

198. Suflita, J.M.; Horowitz, A.; Shelton, D.R.; & Tiedje, J.M.: Dehalogenation: A novel pathway for anaerobic biodegradation of haloaromatic compounds. *Science* 218:1115-1117; 1982.

199. Symons, B.D.; & Sims, R.C.: Assessing detoxification of a complex hazardous waste using the Microtox™ bioassay. *Archives of Environmental Contamination & Toxicology* 17:497-505; 1988.

200. Talbot, H.W.; Johnson, L.; Barik, S.; & Williams, D.: Properties of a *Pseudomonas* sp-derived parathion hydrolase immobilized to porous glass and activated alumina. *Biotechnology Letters* 4: 209-214; 1982.

201. Trulear, M.G.; & Characklis, W.G.: Dynamics of biofilm processes. *Journal Water Pollution Control Federation* 54:1288-1301.

202. Tsien, H.C.; & Hanson, R.S.: Soluble methane monooxygenase component B gene probe for identification of methanotrophs that rapidly degrade trichloroethylene. *Applied and Environmental Microbiology* 58(3): 953-960; 1992.

203. U.S. Environmental Protection Agency: *Bioremediation of Hazardous Wastes*. EPA/600/9-90/044, Biosystems Technology Development Program, Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC; 1990.

204. U.S. Environmental Protection Agency: *Pesticide Treatability Data Base, Version 2.0*. Risk Reduction Engineering Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH; 1992.

205. U.S. Environmental Protection Agency: *Summary Report - High-priority Research on*

Bioremediation. Bioremediation Research Needs Workshop, April 15-16, Washington, DC; April 15-16, 1991.

206. U.S. Environmental Protection Agency: *Symposium on Bioremediation of Hazardous Wastes*. Biosystems Technology Development Program, Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC; May 5-6, 1992.

207. U.S. Environmental Protection Agency: *The Superfund Innovative Technology Evaluation Program: Technology Profiles, Fourth Edition*. EPA/540/5-91/008, Risk Reduction Engineering Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH; 1991.

208. U.S. Environmental Protection Agency: *VISITT: Vendor Information System for Innovative Treatment Technologies*. EPA/542/R-92/001, Office of Solid Waste and Emergency Response, U.S. Environmental Protection Agency, Washington, DC; 1992.

209. U.S. National Research Council: Interdisciplinary research collaboration. *Research News* 37: 27-29; 1987.

210. Vannelli, T.: Logan, M.; Arciero, D.M.; & Hooper, A.B.: Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Applied and Environmental Microbiology* 56: 1169-1171; 1990.

211. Venosa, A.; Haines, J.; Nisamaneepong, W.; Govind, R.; Pradham, S. & Siddique, B.: Efficacy of commercial products in enhancing oil biodegradation in closed laboratory reactors. *Journal of Industrial Microbiology* 10(1): 13-23; 1992.

212. Visscher, K.; Brinkman, J.; & Soczo, E. R.: Biotechnology in hazardous waste management in the Netherlands. Kamely, D., Chakrabarty, A., and Omenn, G.S., editors: *Biotechnology and Biodegradation*. Advances in Applied Biotechnology Series, Volume 4, Gulf Publishing Co., Houston, TX; 1989.

213. Vogel, T.M.: Natural bioremediation of chlorinated solvents. *Ground-Water Cleanup through Bioremediation*. Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK; In press.

214. Vogel, T.M.; Criddle, C.S.; & McCarty, P.L.: Transformations of halogenated aliphatic compounds. *Environmental Science & Technology* 21:722-736; 1987.

215. Vogel, T.M.; Fathepure, B.Z.; & Selig, H.: Sequential anaerobic/aerobic degradation of chlorinated compounds. *Proceedings, International Symposium on Processes Governing the Movement and Fate of Contaminants in the Subsurface Environment*. Stanford University, Palo Alto, CA; July 23-26, 1989.

216. Wackett, L.P.; & Gibson, D.T.: Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. *Applied and Environmental Microbiology* 54: 1703-1708; 1988.

217. Werner, P.: A new way for the decontamination of aquifers by biodegradation. *Water Supply* 3: 41-47; 1985.

218. Westmeier, F.; & Rehm, H.: Degradation of 4-chlorphenol in municipal waste water by

adsorptive immobilized *Alcaligenes* sp. A7-2. *Applied Microbiology and Biotechnology* 26: 78-83; 1987.

219. White, D.C.; Smith, G.A.; Gehron, M.J.; Parker, J.H.; Findlay, R.H.; Martz, R.F.; & Fredrickson, H.L.: The ground water aquifer microbiota: Biomass, community structure, and nutritional status. *Developments in Industrial Microbiology* 24: 201-211; 1983.

220. Wierich, P.: & Gerike, P.: The fate of soluble, recalcitrant and adsorbing compounds in activated sludge plants. *Ecotoxicology and Environmental Safety* 5:161-170; 1981.

221. Wilderer, P.; & Markl, H.: Innovative reactor design to treat hazardous wastes. Lewandowski, G., Armenante, P., and Baltzis, B., editors: *Biotechnology Applications in Hazardous Waste Treatment.* Engineering Foundation, New York, NY; 1989.

222. Wilderer, P.A.; Rubio, M.A.; & Davids, L.: Impact of the addition of pure cultures on the performance of mixed culture reactors. *Water Research* 25(11): 1307-1313; 1991.

223. Wilson, J.T.; McNabb, J.F.; Cochran, J.W.; Wang, T.H.; Tomson, M.B.; & Bedient, P.B.: Influence of microbial adaptation on the fate of organic pollutants in ground water. *Environmental Toxicology and Chemistry* 4: 721-726; 1985.

224. Witholt, B.; Janssen, D.B.; & Keuning, S.: Biodegradation of xenobiotics by specific bacteria: Research and applications. *Biotreatment: The Use of Microorganisms in the Treatment of Hazardous Materials and Hazardous Wastes.* Hazardous Materials Control Research Institute, Silver Spring, MD; 1989.

225. Woodyard, P.T.: Considerations in the selection of environmental biotechnology as viable in field-scale waste treatment applications. Sayler, G.S., Fox, R., and Blackburn, J.W., editors: *Environmental Biotechnology for Waste Treatment.* Plenum Press, New York, NY; 1991.

226. Yakabe, Y.; Etoh, C.; Matsunobu, Y.; Katsuura, H.; Miura, K.; & Yoshimura, K.: Kinetic study on the biodegradation of linear alkylbenzenesulfonates (LAS) in well-water. *Chemosphere* 24(8): 969-977; 1992.

227. Yang, J.; & Speece, R.E.: The effects of chloroform toxicity on methane fermentation. *Water Research* 20(10); 1273-1279; 1986.

228. Zachopoulos, S.; & Hung, Y.: Biokinetics of nitrification in the activated-sludge process with bioaugmentation. *Acta Hydrochimica et Hydrobiologica* 19(6): 683-692; 1991.

Table 1. Overview of Selected Research Regarding Bioaugmentation for the Remediation of Contaminated Aquatic Environments.

Experimental Profile	Specific Contaminants	Treatment Status	Research Summary	Reference
Eight commercial bacterial cultures and two non-bacterial products were tested for their ability to degrade weathered Alaska North Slope crude oil.	Crude oil	Bench scale respirometer and shake flasks	Substantial growth of oil degraders, high oxygen uptake rate, and the degradation of aliphatic and aromatic hydrocarbon fractions led to the decision to implement field testing.	211
A bacterial product containing supplementary heterotrophic and nitrifying microorganisms was introduced in bench scale reactors.	Municipal wastewater	Bench scale; continuous flow activated sludge systems	The results of this study indicate that bioaugmentation improved nitrification at higher wastewater strengths. Bioaugmentation with nitrifying microorganisms could therefore potentially improve the activated sludge treatment of municipal wastewater.	228
The impact of the addition of pure cultures *(Pseudomonas putida PRS 2015 pAC 27)* on the performance of mixed culture reactors was evaluated.	3-chlorobenzoate was used as a model substrate representing chloroorganic pollutants.	Bench-scale bioreactors	Results indicate that the startup times for full-scale reactors may be significantly reduced by the addition of pure cultures.	222

Table 1. (Continued) Overview of Selected Research Regarding Bioaugmentation for the Remediation of Contaminated Aquatic Environments.

Experimental Profile	Specific Contaminants	Treatment Status	Research Summary	Reference
Oil degradation was enhanced by the addition of an oleophilic fertilizer. The oleophilic fertilizer (INEPOL) served to dissolve nutrients into the oil, thus facilitating bacterial growth.	Crude oil	Field-scale	Enhancement of the in situ biological degradation of crude oil using oleophilic fertilizer for nutrients is a promising bioaugmentation application.	162
A variation of the activated sludge process, called an enricher reactor system, was used to degrade polycyclic aromatic hydrocarbons. A second reactor systems is specifically designed to grow cells capable of degrading the target compound.	Naphthalene and phenanthrene	Bench-scale laboratory reactors	Results indicate that this method is a promising way to increase the efficiency of the activated sludge process.	35

Table 2. Overview of Selected Research Regarding Biostimulation for the Remediation of Contaminated Aquatic Environments.

Experimental Profile	Specific Contaminants	Treatment Status	Research Summary	Reference
Growth of an indigenous population of methane-utilizing bacteria was stimulated by the addition of dissolved methane and oxygen into a semiconfined aquifer.	TCE	Controlled field experiments at Moffett Field Naval Air Station, Mountain View, California	Experimental results are lending to model development in order to compare simulated results with those in the field.	175
The presence of nutrients in waters of low redox potential promotes the growth of anaerobic bacteria that reduce nitrate to ammonium.	Dissimilatory nitrate reduction to ammonium is suggested to be a significant process in the study of the fate of solutes in groundwater contraminated from the disposal of treated wastewater on sand beds (7).	Field site studies	Research aim is at developing methods for the addition of biostimulating agents to enhance biological degradation in situ.	30
Phenol is polymerized to form insoluble precipitates (by adding gelatin and polyethylene glycol) to reduse the amount of peroxidase (enzymatic treatment) required.	Wastewater containing phenol, 2-propanol, and 2-butanone	Bench-scale	Research aim is at developing protocols for the addition of agents such as gelatin and polyethylene gylcol, to reduce cost and space requirements for the enzymatic treatment of phenolic wastewaters.	150

Table 2. (Continued) Overview of Selected Research Regarding Biostimulation for the Remediation of Contaminated Aquatic Environments.

Experimental Profile	Specific Contaminants	Treatment Status	Research Summary	Reference
The reductive dechlorination of tetrachloroethene was stimulated in aquifer microcosms by the addition of toluene.	Alkyl benzenes and chlorinated ethenes	Bench-scale microcosms constructed from aquifer solids containing alkyl benzenes and chlorinated ethene's at the U.S. Coast Guard Air Station, Traverse City, MI	Potential full-scale field applications are expected for the future.	179
A microbial consortia enriched from subsurface sediments contaminated with chlorinated hydrocarbons proved capable of degrading mixed-organic wastes.	Mixed-organic wastes (benzene, toluene, xylene, vinyl chloride and TCE)	Bench-scale laboratory bioreactors (continuous-recycle expanded-bed)	Research aim is at the potential bioremediation of groundwater contaminated with mixed-organic wastes.	161
The growth of indigenous methanotrophic microorganisms was stimulated by the addition of methane and oxygen. The resulting microbial population promoted the degradation of certain chlorinated aliphatic compounds.	Trichloroethylene (TCE), cis-dichloroethylene (cis-DCE), trans dichloroethylene (trans-DCE) and vinyl chloride (VC)	Field demonstration	Potential full-scale field applications are expected to incorporate additions of oxygen and methane to enhance biodegradation of mixed chloroorganics.	177

Table 3. Overview of Selected Research Regarding Acclimation for the Remediation of Contaminated Aquatic Environments.

Experimental Profile	Specific Contaminants	Treatment Status	Research Summary	Reference
Linear alkylbenzenesulfonate (LAS) was added to well-water at weekly intervals to evaluate the kinetics of acclimation in well-water.	Linear alkylbenzenesulfonate (LAS)	Field-scale	LAS-acclimated bacteria in well-water multiplied rapidly after the first addition of LAS.	226
A microbial consortia enriched from subsurface sediments contaminated with chlorinated hydrocarbons proved capable of degrading mixed organic wastes.	TCE, benzene, toluene, xylene, and various chlorinated hydrocarbons	Bench-scale continuous-recycle expanded bed reactors	Results indicate the potential for bioremediation of groundwater contaminated with mixed organic wastes.	161
Experiments were conducted to evaluate the effectiveness of selected process strategies with respect to maintenance of the degradative capabilities of added microorganisms.	3-chlorobenzoate (model substrate for chloroorganic compounds)	Bench-scale sequencing batch reactors	The adaptation process was promoted by alternation of periods during which the target substance was the only available carbon source, and during periods which 3-chlorobenzoate was available in combination with favorable growth substrates.	222
Strains of mycobacteria (isolated from activated sludge were correlated with their ability to degrade morpholine.	Morpholine	Bench-scale laboratory activated sludge system	When morpholine was removed from the influent it was observed that the morpholine degrading capacity of the microorganisms was decreased.	29

Table 3. (Continued) Overview of Selected Research Regarding Acclimation for the Remediation of Contaminated Aquatic Environments.

Experimental Profile	Specific Contaminants	Treatment Status	Research Summary	Reference
The potential of a shallow aerobic aquifer to degrade the herbicide Mecoprop (2-(2-methyl-4-chlorophenoxy)propionic acid) was evaluated using ground water and sediment suspensions. Mecoprop was added at increasing concentrations. Acclimation periods ranged from 20-110 days.	Mecoprop (2-(2-methyl-4-chlorophenoxy)propionic acid)	Bench-scale batch suspensions	The adapted microorganisms provided complete degradation after a second lag period in most cases.	99

Table 4. Overview of Selected Research Regarding Genetic Engineering for the Remediation of Contaminated Aquatic Environments

Experimental Profile	Specific Contaminants	Treatment Status	Research Summary	Reference
CYANIDASE® is a new enzyme preparation capable of degrading cyanide in industrial wastewaters.	Cyanide	Bench-scale recirculation fixed-bed reactor, expanded bed reactors or membrane reactors are expected for large scale processes.	Future goal is the development of an enzyme membrane reactor for the processing of cyanide-containing industrial wastewaters without pretreatment.	13
Salicylate degrading bacteria were bred by transferring NAH-plasmid into floc-forming bacteria.	Salicylate	Bench-scale batch cultures	Isolates from the activated sludge (floc-forming recipients) are expected to be used in an advanced wastewater treatment process in the future.	73
A chlorobenzoate-catabolic transposon (Tn5271) was introduced into a natural host, *Alcaligenes* sp. strain BR60	3-chlorobenzoate, 4-chloroaniline, 2,4-dichlorophenoxy-acetate, and 3-chlorobiphenyl	Bench-scale flowthrough lake water and sediment microcosms	*In situ* applications for the degradation of 3-chlorobenzoate and 4-chloroaniline are expected in the near future.	74, 75
A field application vector utilizing 1% Igepal CO-720 (detergent) as a selective substrate and *Pseudomonas paucimobilis* 1IGP4 as the host was tested for its ability to increase the presence of nonadaptive tetracycline resistance marker genes in soil.	Potential applications include: TCE benzo(a)pyrene, and polychlorinated biphenyls	Bench-scale microcosms	The current focus is to develop a field application vector for polychlorinated biphenyl degradation using 1%IGP(detergent)-strain 1IGP4 combination.	121

Table 4. (Continued) Overview of Selected Research Regarding Genetic Engineering for the Remediation of Contaminated Aquatic Environments

Experimental Profile	Specific Contaminants	Treatment Status	Research Summary	Reference
Organisms are genetically engineered to bioluminesce for monitoring applications in the environment.	N/A	Small-scale field introduction	Future work aimed at developing methods to examine the movement and persistence of genetically engineered organisms in the environment.	180
Isolation and sequencing of the dissimilatory nitrite reductase gene (*nir*) from denitrifying bacterium *Pseudomonas stutzeri* JM300 provides a method for evaluating the population of denitrifying bacteria in the environment.	N/A	Non-treatment monitoring method successful in detecting denitrifying bacteria from samples such as a bioreactor consortium, aquifer microcosms and denitrifying toluene-degrading enrichments.	Research focus is on the development of a fast and economical method to screen for the presence of denitrifying bacteria in various environmental communities.	190
Restriction fragment length polymorphisms, Western blot (immunoblot) analysis, and fluorescence-labelled signature probes were utilized for the characterization of methanotrophic bacteria in addition to the identification of methanotrophs containing the soluble methane monooxygenase (MMO) gene and capable of degrading trichloroethylene (TCE).	N/A	Non treatment monitoring method successful in determining the presence of bacteria that produce soluble MMO in bioreactors and environmental samples.	Research focus is on the development of a fast and economical method to screen for the presence of bacteria that produce soluble MMO, indicating the organisms ability to degrade TCE.	202

Table 5. Types of Bioengineering Treatment Systems.

Bioengineering Technique	Description
Attached-Growth	Biomass attached to immobile carrier media within the bioreactor.
Aerobic Bioreactor Systems	Oxygen used as the terminal electron acceptor.
Trickling Filters	Media consists of crushed stone or rock; Contaminated water dosed on surface of media and is degraded as it "trickles" through the media.
Rotating Biological Contactors	Media consists of corrugated disks mounted on a central shaft, which is mounted lengthwise in a horizontal cylindrical tank, which holds the contaminated water; Disks rotate slowly, with about 40 % submerged in water; Water, as disks rotate, is moved out into the air for aeration; Microorganisms form biofilm on disks.
Aerobic Fluidized Bed Reactors	Media consists of sand particles suspended in a vertical column by upward flow of contaminated water; Flow adjusted so sand particles are kept in motion but are not swept out of the system; Addition of air or oxygen required.

Table 5 (Continued). Types of Bioengineering Treatment Systems.

Bioengineering Technique	Description
Attached-Growth	Biomass attached to immobile carrier media within the bioreactor.
Anaerobic Bioreactor Systems	Fermentation reactions used for degradation processes; Bioreactor systems are closed.
Anaerobic Filter	Media consists of gravel, rocks, or plastic media; Reactor operated in an upflow mode.
Anaerobic Fluidized Bed Reactor	Media consists of sand particles suspended in a vertical column by upward flow of contaminated water; Flow adjusted so sand particles are kept in motion but are not swept out of the system.
In Situ Ground-Water Treatment	Contaminated ground water treated in aquifer
Aerobic with Indigenous Microorganisms	Biostimulated with addition of nutrients, oxygen or oxygen source (e.g., hydrogen peroxide), and/or primary cometabolic substrates.
Aerobic with Introduced Microorganisms	Bioaugmented with acclimated , selected, or genetically engineered microorganisms as well as biostimulated with nutrients, oxygen or oxygen source, and/or primary cometabolic substrates.

Table 5 (Continued). Types of Bioengineering Treatment Systems.

Bioengineering Technique	Description
Attached-Growth	Biomass attached to immobile carrier media within the bioreactor.
In Situ Ground-Water Treatment	Contaminated ground water treated in aquifer
Anaerobic with Indigenous Microorganisms	Biostimulated with addition of nutrients, alternate electron acceptors, and/or primary cometabolic substrates.
Anaerobic with Introduced Microorganisms	Bioaugmented with acclimated , selected, or genetically engineered microorganisms as well as biostimulated with nutrients, alternate electron acceptors, and/or primary cometabolic substrates.
Drinking Water Treatment	
Slow Sand Filtration	Media consists of fine sand particles. Biofilm forms as mat on top of sand bed (referred to as schmutzdecke). Schmutzdecke composed of algae, bacteria, protozoa, and rotifers. Filters are operated at low filtration rates, resulting in long retention times.

Table 5 (Continued). Types of Bioengineering Treatment Systems.

Bioengineering Technique	Description
Suspended-Growth	Biomass is suspended in the liquid phase of the bioreactor.
Aerobic Bioreactor Systems	Oxygen used as the terminal electron acceptor.
Activated Sludge	System consists of aeration basin to which contaminated water is introduced, along with air or oxygen. Flocculated microorganisms are kept in suspension and mixed by the aeration process. Microorganisms that are removed in the effluent are separated from the effluent by gravity settling; Part are returned to the aeration basin; Part are removed as biological sludge for disposal; Retention time is hours.
Waste Stabilization Lagoons	System consists of a basin to which contaminated water is introduced. Bacteria utilize organic carbon present in wastes, producing new cells and carbon dioxide; Carbon dioxide is utilized by algae and blue-green bacteria, which produce oxygen that is used by the bacteria. Retention time for treatment is weeks.

Table 5 (Continued). Types of Bioengineering Treatment Systems.

Bioengineering Technique	Description
Suspended-Growth	Biomass is suspended in the liquid phase of the bioreactor.
Anaerobic Bioreactor Systems	Fermentation reactions used for degradation processes; Bioreactor systems are closed.
Anaerobic Contact Process	System consists of continuously stirred tank reactor (fermenter) containing an active population of flocculated bacteria, followed by a settling tank. Microorganisms that are removed in the effluent are separated from the effluent by gravity settling; Part are returned to the aeration basin; Part are removed as biological sludge for disposal.

Table 6. SITE Program Participants with Bioremediation Technologies Applicable to the Treatment of Contaminated Water Sources.[207]

Technology	Description	Applications	Developer
Immobilized Cell Bioreactor	Aerobic fixed-film bioreactor utilizing a proprietary media that maximizes biological activity and a proprietary design that maximized contact between biofilm and contaminants.	Organic compounds, including nitrogen-containing compounds and chlorinated solvents; suitable for removal of low levels of contaminants.	Allied-Signal, Inc. Morristown, NJ
Biological Aqueous Treatment System	Multiple-cell, submerged, fixed film bioreactor using a microbial population indigenous to the waste-water to which a specific has been added; may include pH adjustment, inorganic nutrient addition, heating, and aeration; may also be run under anaerobic conditions.	Ground water, lagoons, and process waters containing creosote, gasoline, fuel oil, chlorinated hydrocarbons, phenolics, solvents, and pentachlorophenol.	BioTrol, Inc. Chaska, MN
Methanotrophic Bioreactor System	Continuous flow, dispersed growth bioreactor using the enzyme methane mono-oxygenase to co-oxidize halogenated aliphatic hydrocarbons.	Water contaminated with halogenated aliphatic hydrocarbons.	BioTrol, Inc. Chaska, MN
On-site Bioreactor	Above-ground bioreactor for treatment of contaminated ground water.	Chlorinated solvents and non-chlorinated organic compounds.	Ecova Corp. Redmond, WA
PACT® Wastewater Treatment System	Aeration basin containing biomass for biodegradation and powdered activated carbon for removal of remaining organic compounds activated carbon; may include temperature and pH control and nutrient addition.	Municipal and industrial wastewaters, ground water and leachates containing organic compounds.	Zimpro/Passavant Environmental Systems, Inc. Rothschild, WI
Chemical and Biological Treatment	Chemical oxidation, using hydroxyl radical, to produce transformation products that can be biologically degraded, followed by biological treatment using aerobic and anaerobic bioreactors either in sequence or alone.	Organic pollutants in water, including alkenes, chlorinated alkenes, aromatics, and substituted and complex aromatics.	Institute of Gas Technology, Chicago, IL
Membrane Separation and Bioremediation	Filtration unit for concentration of contaminants from water and bioreactor containing proprietary microorganism mix for treating concentrated contaminants.	Organic compounds, including PAHs. creosote, PCBs, oily wastes, jet and diesel fuels, and trichloroethylene.	SBP Technologies Inc. Stone Mountain, GA

Table 6 (Continued). SITE Program Participants with Bioremediation Technologies Applicable to the Treatment of Contaminated Water Sources.[207]

Technology	Description	Applications	Developer
Two-Zone Plume Interception In Situ Treatment Strategy	Ground water treatment in which partial dechlorination is accomplished in first zone by stimulation of methanogenic bacteria with addition of primary carbon source (e.g., glucose) and mineral nutrients; in second zone, in which oxygen is added, transformation by-products of first are degraded by aerobic methanotrophic bacteria utilizing oxygen and methane.	Ground water containing chlorinated and non-chlorinated solvents.	ABB Environmental Services Wakefield, MA

Table 7. Types of Anaerobic Bioreactor Systems.

System
Anaerobic Contact Process
Description
Consists of continuously stirred tank reactor (fermenter) containing an active population of flocculated bacteria, followed by a settling tank (clarifier). SRT greater than HRT is accomplished by settling solids from the effluent in the clarifier and recirculating a concentrated sludge back to the fermenter (similar to aerobic activated sludge process).
Applications/Advantages/Limitations
Since bacteria are retained and recycled, can treat medium strength wastewaters (i.e., containing CODs of 2,000 - 20, 000 mg/L). However, can be difficulties in recycling bacteria.
Anaerobic Filter
Description
Consists of a reactor filled with an inert support material with a high surface area, such as gravel, rocks, or some plastic media. Reactor is operated in the upflow mode. Long SRTs and high hydraulic loading rates are possible. Once established, reactors are resilient to variable loading rates and moderate environmental changes such as pH or temperature.
Applications/Advantages/Limitations
Has outstanding ability to retain biomass. May have accumulation of solids in the columns, if contaminated water is high in suspended solids. May require frequent blowdown of the column to remove solids.

Table 7. (Continued) Types of Anaerobic Bioreactor Systems.

System
Anaerobic Fluidized Bed Reactors
Description
Consists of compact, tall, and slender reactors filled with small, heavy particles, which act as physical supports for the growth of biomass. These particles that are covered with biological growth are maintained in a fluidized state by an upwards-directed flow of water. Due to the heavy particles, the settling velocity is very high, and high liquid velocities can be maintained in the reactors, Biomass concentrations are very high due to the large surface area of the small particles (e.g., sand).
Applications/Advantages/Limitations
The high liquid velocities prevent the accumulation of solids. Because of the high biomass concentrations and activity, a high treatment capacity is obtained. Reactors are compact and require little space. However, systems may have a long start-up period due to problems with the establishment of methanogens on the solid supports. More research is required to identify (1) optimal choice of particles (e.g., composition and size), (2) characteristics of waste water that affect biolayer development, (3) operational characteristics that affect biolayer attachment, and (4) factors that control the development of optimal biolayer thickness (i.e., the biolayer should be sufficiently thick to give a biomass concentration, but sufficiently thin to avoid washout from the reactor. Additional limitations include high energy consumption due to very high liquid recirculation ratios and difficulties with maintenance of the fluidized bed.
Upflow Anaerobic Sludge Blanket Reactor (UASB)
Description
Consists of an upflow reactor with an internal baffle system for separation of gas, sludge, and liquid. With the baffle system, gas is separated from the sludge, collected under the plates, and piped away. Above the plates is a relatively quiet zone, where the sludge is separated from the fluid and can settle back towards the digesting zone. Reactor is primarily mixed by the gas production rather than forced mechanical mixing.
Applications/Advantages/Limitations
Reactor accommodates well to hydraulic and organic shock loadings, temperature fluctuations, and low influent pH values, as long as the reactor pH stays above pH 6.0. Adequate sludge residence times and high anaerobic sludge concentrations can be maintained. Sludge retention may be a problem if granular sludge is not obtained.

Table 8. Examples of Costs for Typical *In Situ* Systems for Aerobic Bioremediation of Ground Water Using Indigenous Microorganisms.[153]

Site Location/Area of Contamination	Type of Contamination	System Type	Estimated Costs
New York/ 1 acre	Gasoline	Infiltration trench for nutrients and hydrogen peroxide; three 80 gpm	$250K
Iowa/ Not given	PAHs and BTEX	*In situ*	$1.65M
Kansas/ 700K cubic feet	BTEX	*In situ* with nitrate as electron acceptor; combined with soil flushing	$925K
California/ 3,000 cubic yards	Diesel and gasoline	Closed loop system with hydrogen peroxide as oxygen source	$1.6M
Michigan/ 1/4 acre site	Gasoline	Infiltration gallery and injection wells, with air and hydrogen peroxide as oxygen sources	$600K
Texas/ 20 acres	BTEX, chlorinated solvents, other organics	*In situ* bioremediation, with oxygen and nitrate as electron acceptors; combined with pump & treat in above-ground bioreactor	$5.325M