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Introduction

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The effects that pollution is having on our environment and ourselves is becoming more evident as statistics describing changes in ecological and health effects indicators are gathered and analyzed. These effects are manifested by their negative impact on tree stands in forests around the world, water and air purity, and other specific indicators of ecological balance, and by their impact on human health. Some of these problems are associated with the production of products used in our daily lives. Wastes generated by producers of autos and related petroleum products, industrial chemicals, pesticides, plastics, paper, etc., have been and still are being placed in dump sites around the world. Many of these effects are the result of emissions from industrial facilities, urban populations (e.g., autos, space heaters) and agricultural sources (pesticides, fertilizers). These pollutants have diverse impacts, ranging from the well known (global warming, depletion of ozone layer) to the not so well known (groundwater depletion and spoilage) (Council on Environmental Quality 1979). Many of these wastes are directly toxic to humans and hazardous to the environment.

Most of the waste is disposed of in landfills, stored in containers, or simply dumped on the ground. This practice has been going on for decades, resulting in the existence of many filled sites containing unidentifiable containers and residues. There are approximately 14,000 industrial sites in the United States (US) producing about 265 tons of hazardous waste annually. Table One lists the common types of wastes found in typical waste dumps in the US. As can be seen, the volume and type of waste material varies greatly, as does the toxicity. More than

6,000 sites have been cleaned up since 1982. In The Netherlands costs of soil rehabilitation efforts in the European community are expected to reach $10 billion by the year 2000 and reach $30 billion over the following decade (Porta 1991).

The solution to the problem is not clear. Choices must be made between methodologies for site cleanup and decisions as to which sites are to be treated first and by what means. The choice of techniques is limited to physical (e.g., incineration, immobilization), chemical (e.g., neutralization), or biological (use of natural or engineered microbes), each of which have specific advantages and disadvantages (e.g., costs, safety issues, time to completion).

The use of microbes to degrade waste is not new. Man has been using microbes to treat sewage wastes for centuries and the process is still being improved upon (Nicholas 1989, Mckinney 1962, Sterritt and Lester 1988). With the advent of biotechnology biological techniques are being reexamined and improved, and with improvements in genetic engineering techniques altered microbes are available to more rapidly degrade noxious materials.

It must be kept in mind that it is not sufficient to develop technical solutions that can be demonstrated in the laboratory. Technical issues are only one part of the solution. Regulatory issues, economics, safety considerations, business and market issues, and social and political considerations all play major roles in the application of technically possible solutions.

This chapter will describe the development and use of microbes to degrade hazardous

waste, and briefly review procedures for degrading common wastes. Potential health and ecological problems associated with commercial scale applications will be identified and mitigation and control methods discussed.

Biodegradation

Biodegradation is the process of mineralization of organic material by microbes. This environmental process has been known for centuries. Organic matter is cycled from organic to mineral material through the action of microbes acting in a cyclical manner (Marx 1989). Organic compounds are reduced to CO_2 and H_2O either via aerobic or anaerobic metabolism. In the anaerobic process, CH_4 is produced. In the carbon cycle atmospheric carbon dioxide is incorporated into organic compounds by photosynthetic organisms. In the sulfur cycle sulfur processes inert sulfur and organic sulfur containing compounds. In both cycles tons of material are changed on an annual basis as a result of microbial action. It has been estimated that 6,000 tons of sulfur pass through the cycle annually.

Over the millennia that microbes, plants, and mammals have coexisted microbial capability to degrade (decompose) organic matter has evolved in parallel to the ability of plants and animals to produce different types of organic matter. However, the first synthesized organochloride compound, ethyl chloride, was prepared in 1440 and large scale synthesis of chlorinated organic compounds at commercial levels has occurred only for the past few decades. This short time frame has not permitted the evolution of microbial systems capable of rapidly and

facilely coping with the onslaught of xenobiotic chemicals (Hutzinger and Verkamp 1981; Rochkind et al. 1986). Thus many xenobiotic chemicals are resistant to microbial attack and/or are toxic to the microbes, hampering man's attempts to harness this ability. Nevertheless, microbes that can degrade many xenobiotic compounds with different degrees of ease and at different rates (Table Two) have been isolated from locations contaminated with various xenobiotic chemicals. As can be seen, the environmental isolates vary greatly in ability to degrade congeners of chlorinated aromatic compounds. Some can degrade more than one compound and do so at different rates. Abramowicz (1989) demonstrated similar results in soils contaminated with polychlorinated biphenyl (PCB) compounds. Twenty six isolates degrading a wide variety of PCB congeners were found. He proposed combining the genetic capabilities to produce a single more useful microbe.

Some compounds are mineralized by a mix of organisms. This fact results in the use of a mix (consortium) of natural isolates that has the capability of degrading a number of target compounds. In some cases identification of the microbes in the consortium has been accomplished, but in many cases a consortium containing an unknown number of unidentified microbes is used. A consortium of microbes may be involved in the sense that specific microbes may be needed for the range of specific components of the waste mixture or in the sense that a combination of microbes may be needed for a particular compound (Table Three). Some microbes are active only as a member of a specific pair. In this process (cometabolism) the compound being degraded serves as an energy or carbon source (Atlas and Bartha 1987). The work of Pfeander and Alexander (1977) and of Sakazawa (1981) illustrate the fact that where

cometabolism is involved, often the species designation of the organism(s) involved is not known, although in most cases the genus is specified. When consortia of microbes are involved, the end products of metabolism are identified and the microorganisms are often not specified (Fliermans et al. 1988, Nielson et al. 1987). It has been suggested that whatever man can make, nature can degrade (Sterritt and Lester 1988).

Microbes are sensitive to environmental conditions. In general, acidity or alkalinity in the neutral range is optimum and temperatures close to normal body temperature are optimal. However, microbes are active at extremes of temperature (psychrophilic and thermophilic bacteria) and these characteristics are being exploited for use in specific waste treatment situations. It has been estimated that efficiencies of psychrophilic bacteria are 60 to 70% of the mesophiles (Bioremediation Report 1991). It must be kept in mind that microbial metabolism is susceptible to shifts in environmental conditions, build up of intermediate metabolic products may occur, and some of these products may be more toxic than the original material. Tetrachloroethylene (a known animal carcinogen) degradation can result in the accumulation of vinyl chloride (a known human carcinogen) under conditions of anaerobic degradation (Barrio-Lage et al. 1986). McCall et al. (1981) reported that during the degradation of 2,4,5,Trichlorophenoxyacetic Acid, in addition to CO_2, concentrations of 2,4,5, trichlorophenol and 2,4,5 trichloroanisole were found in the soil.

Research is being conducted in many laboratories to enhance the degradative ability of natural microbes (Rojo et al. 1988) while others are attempting to create altered microbes with

enhanced degradative scope and rates. Rojo has demonstrated the integration of enzymes from five different catabolic pathways of three different, distinct, soil bacteria into one strain. Attention is also focusing on isolates that can survive and flourish after being released at a waste site (Dwyer et al. 1988, Neidle et al. 1987). Some researchers are attempting to better understand the environmental parameters that control the metabolic rates and genetic composition of microbial flora in situ (Olson and Goldstein 1988) with the objective of manipulating environmental parameters to enhance selected degradative characteristics of the natural flora. The US National Science Foundation conducted a workshop to discuss the feasibility of field applications of environmental biotechnology (Sayler et al. 1988). Although no large scale field applications of engineered microbes have been conducted, tests on mutated isolates have been conducted and tests are planned for engineered strains (Bioremediation Report 1991).

Attempts to more fully exploit the degradative ability of microbes on a commercial scale take several forms. The oldest and most direct is the enhancement of sewage treatment by modification of the treatment process (Hall and Melcer 1983). Mizrahi (1989) reviewed the various treatment methods and the modifications in biogas digestors, anaerobic digestion technology, and managerial aspects that result in more efficient sewage plant operation. All of the sewage treatment methods involve three components: physical manipulation of the environment, chemical augmentation of the microbial nutrient mixture, and augmentation of the microbial population (either adding additional natural organisms by engineering a microbe with superior performance characteristics, or by encouraging the growth of indigenous microbes by adding appropriate nutrients). The development of biotreatment begins with the development of

methods to accelerate the rate of degradation of sewage and to obtain effluent less harmful to human health and the environment. Early treatment of sewage consisted of sprinkling it on large areas of land (0.4 ha - 1 acre - per 100 persons). Studies at the Lawrence Experimental Station in Massachusetts in 1889 lèd to the use of gravel as a percolating filter. This work was followed by the development of anaerobic digestion and then aerobic digestion of sewage at Davyhulme, Manchester. Aerobic digestion, which is simply adding air to the digestion mixture, when combined with the use of an inoculant from previous digestions shortened the digestion period from five weeks to twenty four hours (Sterritt and Lester 1988). This is perhaps the earliest example of using naturally adapted microbes to enhance the degradation of waste material.

These features are common to all methods being developed for use in treating pollution problems at dump sites or spill locations. Many different procedures have been developed to permit and enhance contact between microbes and the target pollutant. Table Four describes some of the most common procedures and the safety issues associated with each. Clearly, use of immobilized microbes or fixed film bioreactors will result in minimizing release of microbes to the environment, thus minimizing the possibility of adverse environmenal or health effects. In addition, any type of reactor can be combined with appropriate sytems for disinfecting the effluent to assure containment of the microbes involved in the process. Soil treatment systems and applications involving subsurface reclamation or land farming will result in extensive dispersal of microbes. In these situations, emphasis must be placed on assuring that the microbe(s) is innocuous.

In general, experience has shown that no one component is ideal for all sites and that some combination is essential to obtain optimal degradation. In addition, at some polluted sites some form of physical or chemical pre or post treatment may be necessary.

Over 100 US companies are actively engaged in applying scaleup procedures to biodegradation techniques to clean up waste sites. Most are also involved in research to improve the biodegradation process without the use of engineered organisms. Major firms, such as Dow Chemical and General Electric, are involved in developing and implementing methods for the biotreatment of wastes. A group of companies has formed an association and produced a compendium describing successful instances of biotreatment on a commercial scale (Applied Biotreatment Association 1989). Microbs have been employed successfully to clean up some of the Alaskan coastline after the Exxon Valdez spill (Crawford 1990) as well as at locations in the US and Europe (Savage 1987, Bluestone 1986, Stone 1984, Keeler 1991). They have also been used to control odors from treatment plants (Grubbs and Molnaa 1987). To date only nonengineered isolates have been used as inocula. In many cases (e.g., the Exxon spill) treatment consists of adding nutrient material to enhance the growth of indigenous microbes. The use of engineered microbes offers the possibility of faster degradation of a broader range of compounds. However, the engineered organism may not persist as well in environmental situations (Lenski 1991) and may not survive long enough to accomplish the objective. In addition there is public resistance and hence governmental resistance to the environmental application of engineered microbes. When these considerations are balanced against the availability of naturally occurring microbes, alone or in consortia, to mineralize most target

compounds the basis for commercial emphasis on the use on naturally occurring microbes is clear.

Procedures involved in on site biodegradation

It is now generally accepted that the application of biotreatment must include a thorough hydrological and physical analysis of the site involved as well as laboratory and field studies to determine the appropriate strategy and to determine the need for some form of physical or chemical pre or post treatment. The physical aspects of the site must be determined from the perspective of the effect on the metabolism of the microbes to be added or nurtured. The native microflora must be examined for degradative capability and for nutrient requirements. Finally, the degradative process must be successfully demonstrated in the laboratory and also demonstrated to be effective on a large scale (Wick and Pierce 1990). This holds whether the treatment is to be in situ, in that the material to be treated is not moved from its location and is to be treated by altering the moisture content, nutrients, or microbial flora at the site under natural conditions, or whether the treatment is to take the form of transferring the material to be treated to reactors in which exposure to microbes under controlled conditions will occur. If the treatment is to be conducted in situ, monitoring procedures, including the selection of the compounds to monitor, sampling times and locations, and duration of the monitoring period must be established before the project begins. Cost and regulatory considerations must be taken into account before finalizing the treatment process.

Site examination

A complete survey leading to a thorough understanding of the waste site is essential for the success of the project. This includes characterization of the waste and the site. The type of waste material will govern the choice of microbes and the need for physical or chemical treatment. The type of soil and hydrology involved at the specific site will govern both the schedule for addition and the need for nutrients and moisture. One to two years can be required for site evaluation. Keystone Environmental Resources spent two years studying the soil beneath and immediately adjacent to a contaminated area (Campbell et al. 1989). During this time the physical aspects, such as site hydrology, soil type, subsurface conditions, and climate characteristics were defined while laboratory studies to determine the characteristics of the microbial flora and the impact of the pollutants on the flora were carried out.

Results of feasibility trials showed that the microbes present at the site could degrade the contaminating material if appropriate nutrients and moisture were supplied. The Keystone project involved the addition of nutrients (nitrogen, phosphorous, and minerals) and nitrate as an alternate electron acceptor. Typically, to degrade approximately 1,000 gallons of hydrocarbon material, 10,000 lbs of oxygen and 875 lbs of ammonia nitrogen would be required, resulting in the production of approximately 7,000 lbs of bacteria.

A sampling procedure was developed to provide monitoring of both the success of the treatment and the level of nutrient available. In this case, the chloride content was monitored

as an indication of mineralization and direct pollutant measurements were made at three upstream and three downstream wells. After 12 weeks of treatment, approximately 90% of the contaminant had been removed. In other field applications, a 98-99% reduction in the levels of carbon tetrachloride, chlorobenzene, ethyl benzene, toluene, 1,1,1 trichloroethylene and xylene have been achieved.

Identification, to the species level, of the microbes involved is not commonly attempted. The degradation process often involves a consortium of microbes, including strains in the genera Nocardia, Pseudomonas, Acinetobacter, and Flavobacterium. Biodegradation is often the result of the metabolic activity of a group or consortium of microbes. One company reports that as many as thirty two different microbes were involved in degrading a specific gasoline spill (Bluestone 1986). In general, the more complex the mixture the more complex the consortium of microbes (Bluestone 1986, Olson and Goldstein 1988). Research to better understand the relationship between the genetic capability of the entire microbial population at a given site and the phenotypic expression of biodegradation (Olson and Goldstein 1988) is ongoing. The intent is to develop methods to identify and augment, in situ, the specific genes that contribute to the degradation of specific compounds rather than provide enough nutrients to result in general microbial growth. This will require a much deeper understanding of the factors controlling gene expression and multiplication under environmental conditions and may lead to less expensive, more rapid degradation of wastes with less potential for adverse environmental impacts. For more recalcitrant wastes modified organisms may be developed or the use of some form of bioreactor will be required.

To date, no engineered organisms have been used in in situ situations involving release to the environment, because of regulatory considerations. Modified microbes have been used in bioreactors. Bioreactors provide containment of the microbes, thus avoiding some of the environmental issues. In addition they provide control over the physical conditions of the biodegradation process. The temperature, time of contact with the microbes, nutrient levels, and concentration of the material to be degraded can be optimized. The use of Sequencing Batch Reactors (SBR's) to treat leachate is described by Wick and Pierce (1990) and by Irvine et al. (1982). Irvine's efforts focused on an leachate from a contaminated industrial site. Initially the leachate was placed in storage tanks in contact with "nonsterile raw waste feed" from a wastewater plant for up to 19 days prior to being filtered through granular activated carbon (GAC) columns. Modified organisms were added to the reactors.

Ultimately a neutralization step coupled with the augmentation of the microbial population by the addition of pure cultures isolated from the indigenous population was instituted (Wick and Pierce 1990). A unique strain of Pseudomonas putida that was uniquely adapted to the SBR environment and possessed degradative abilities not found in the original strains, was isolated, cultivated, and added to the existing microbial mix in the SBRs. The SBRs were operated as closed systems. All volatile organic material was trapped on GAC and recycled through the SBRs.

Table Five lists the parameters monitored to permit evaluation of the efficacy of the process and to assure regulatory compliance. The intensive monitoring effort requires the careful

selection of locations, times, and handling of sample material. The analytical methods used to estimate the concentrations of compounds under regulatory control must be acceptable to the regulatory agency and appropriate quality assurance procedures are required. This is an essential and expensive part of any biotreatment project.

The SBRs were operated on a 24 hour cycle. The annual treatment volume was in excess of 10^4 cubic meters. Reduction of monitored compounds varied greatly. Chlorobenzoic acid (ortho and meta) was not detectable (sensitivity of measurement: 3.5 mg/l) with starting levels of 763 and 219 mg/l, respectively. Total organic halide levels were reduced from 1,062 to 319 mg/l (70%). The SBR process had the greatest effect on TOC and phenol, achieving greater than 99% reduction from starting levels of 10,575 and 1,553 mg/l, respectively. The SBR treated leachate still required GAC treatment to meet discharge standards. However, because of the biotreatment, the amount of carbon needed was dramatically reduced. Carbon filters replacement shifted from an every day procedure to approximately 3 times per year. The cost reduction was calculated at approximately $30 per cubic meter of water treated.

Other types of batch reactors include the use of microbes and/or enzymes attached to a support material. In this procedure, the reactor serves as a packed column through which the liquid to be treated is passed. Figure One illustrates such a system, in this case developed by Biotrol Inc., and used in the USEPA Superfund Innovative Technology Evaluation program (USEPA SITE, 1988, Ellis and Stinson 1991). The units can be operated aerobically or anaerobically and permit control of the temperature, retention time, conditioning of the waste

liquid (pH, nutrient adjustment), and convenient monitoring of the influent and effluent. The microbial population can be altered to permit degradation to treat a broad spectrum of contaminants. The company has also developed a soil scrubbing procedure to release bound material to the liquid phase permitting treatment in the bioreactor.

Many variations of a few basic procedures have been developed and reported on for the biotreatment of contaminated soil and water. These include the use of proprietary equipment, cultures, and nutrient formulations. Table Four describes the basic procedures that have been applied commercially to treat liquids and soils under contained, controlled conditions and in situ. As can be seen, these can be divided into two basic types, bioreactors that involve some type of liquid/microbe interaction and soil treatments in which the contaminating material is treated while still adsorbed to particles. The bioreactors generally involve soil washing in which desorption of the target compounds is accomplished by treatment with solvents or a specific, often proprietary, washing solution. The liquid is then treated by exposure to microbes in digestion tanks (SBR) or in aqueous treatment systems or fixed film bioreactors where the microorganisms are attached to some form of support and the liquid is passed through. Soil slurry systems and land farming involve in situ mixing of soil, nutrients, and moisture in various proportions to acheive maximum contact between the microbe and the target compounds.

Methods to maximize contact between microbes and the material to be treated include solubilizing the material and increasing the exposure area by using any of a variety of physical media providing attachment surfaces. The use of enzymes in "immobilized" systems has been

proposed. The contaminated liquid would be pumped through a column containing an immobilized enzyme that would catalyze one step in the biodegradation process. The cost and efficacy of this approach have not been established.

Soil systems, such as the Keystone project, rely on nutrient and moisture addition with constant tilling to provide contact between the microbes and the material to be digested. Environmental parameters (pH, temperature) are manipulated to maximize the reaction rates and end products produced. Existing microbial populations are augmented by adding cultures of microbes grown in the laboratory (either as taken from the site or after selection for specific degratory characteristics).

Treatment rates vary greatly depending on the type of material, physical characteristics of each site, goal of the operation in terms of acceptable final concentrations of pollutants and scale selected for the operation. Reported rates range from 60,000 gallons per week of leachate to 1.7 tons of soil per month. One bioreactor is being used to treat 700-1,000 lbs of cyanide residue from a steel-coking operation daily (McCormick 1985).

The proceedings of the Hazardous Materials Control Research Institute symposium on biotreatment (1989) and the USEPA SITE report (1988) contain detailed descriptions of specific techniques. Most of the techniques demonstrated significant removal of pollutants. Generally 80-98% of the compound(s) being monitored was removed. Although in some cases complete removal is not achieved, the volume of material requiring treatment is significantly reduced,

providing large cost and time savings.

Cost

Cost figures can be found in a number of sources (Bluestone 1986, McCormick 1985, Savage 1987, Rishel et al. 1984, W ck and Pierce 1990). However, comparison of costs between various modes of remediation is difficult because one must take into consideration more than the direct estimation of actual expenses. Each process has advantages and disadvantages and costs vary greatly. The type of material and site characteristics are major factors. Table Six compares cost per cubic yard, time required for a large project, and a few of the major considerations. Biotreatment is the least costly if one considers only immediate cost. It requires the least energy and can result in mineralization of the waste material to innocuous products. However, biotreatment takes longer and does not necessarily result in cleanup to the level required by federal or local regulations. This fact may result in the need for additional treatment and additional cost. Biotreatment results in signifcant reductions in volume of the waste, reducing the cost of follow-up treatments.

Health and Environmental Hazards

Background

Under ideal conditions all biodegradation attempts would result in mineralization of the

target compounds. Aerobic processes would yield carbon dioxide and water, and anaerobic processes would yield methane and inorganic ions. As indicated above, biodegradation is not a new process. However, the use of engineered microbes to enhance the process and more widespread use of biological methods for the treatment of wastes have raised some risk assessment issues not previously considered and have placed greater emphasis on existing issues.

Assessing the risks associated with environmental applications of engineered and natural microbes has been the subject of active research over the past decade. There is general agreement that the estimation of risk involves the identification and quantification of the hazards involved and a coupling of that information with the exposure factor. Numerous authors and organizations have suggested procedures and protocols for evaluating the risks associated with the environmental application of engineered or natural microorganisms (Levin and Strauss 1991, Ginzburg 1991, Office of Technology Assessment 1985, Tiedge et al. 1989, National Academy of Sciences 1989). There have been debates over the issues involved in risk assessment (Sharples 1987, Davis 1987). Methods to monitor and control the microbes have been developed and reviewed in general (Levin et al. 1987, 1992, Biotechnology Action Programme 1990, OECD 1986) and specifically (Vidaver and Stotzky 1992, Vandenbergh 1992, Lindow et al. 1992, Katz and Marquis 1991, Sharples 1991).

There is general agreement that utilization of approaches to the degradation of waste material involving biotechnology will result in more complete mineralization of the target material at less cost in terms of energy utilization. However three types of problems are

recognized when considering the environmental application of microbes for waste treatment: generic problems associated with the use of microbes (engineered or natural isolates), problems associated with the microbial process of degradation of waste material, and specific problems associated with uncontained techniques to enhance the rate of microbial degradation. There is also general agreement as to the information needs to assess the risks associated with the environmental application of engineered or exotic (i.e., nonindigenous) microbes.

Generic Problems Associated with Using Microbes to Degrade Wastes

The environmental application of chemical products is well accepted and methods to assure safety have been developed and proven over the past decades. Many of the concerns identified with the environmental application of microbes are similar and initial attempts to deal with health and safety issues have been based on methodologies developed to assess the risks associated with the use of chemicals in environmental situations. Milewski (1985) defined the problems associated with the field testing of engineered microorganisms and presented a list of points to consider in evaluating a proposed field application. These included:

1. Genetic Considerations: Identification of the parental organism, the host organism, and the genetic material to be transferred as well as information describing the construction of the modified organism, the means of transfer, and the stability and expression of the introduced material.

2. Environmental Considerations: Information about the organism to be modified including habitat and general distribution, as well as survival, reproduction, and dispersal characteristics; a discussion of biological interactions to indicate host range, interactions with other organisms, possible impact on biological cycling processes, and likelihood of exchange of genetic information with other organisms in nature.

3. Field Test Information: Description of the proposed test (objectives, significance, and justification) and any relevant laboratory data describing survival, replication, and dissemination of the modified organism; a description of the conditions of the field test including numbers of organisms, location, specific target organisms that would be affected and methods to contain and monitor the trial.

These points have been reemphasized over the years (Sharples 1991, US General Accounting Office 1988) and respond to five main issues: 1) Will the organism survive ? 2) Will it multiply? 3) Will it spread to other sites? 4) Will it be harmful? and 5) Will it transfer genes to other nontarget organisms? The National Academy of Sciences (1987) summarized the problem by stating that the "assessment of the risks of introducing engineered organisms into the environment should be based on the nature of the organism and the environment into which it is introduced". Subsequently, the issues of decontamination and mitigation have been raised (Vandenbergh 1992).

Although most of the early emphasis was on agricultural applications, these generic safety

issues apply equally to the introduction of microbes (engineered or natural) for waste treatment purposes. Since risk is a function of hazard and exposure the responses to the above questions provide a basis for assessing the risks involved in using a microbe in a particular environmental situation.

Problems Associated with Microbial Degradation of Wastes

Health Issues

Two distinct health issues are involved when assessing risks associated with biotreatment. These are 1) possible effects on workers and 2) possible public health effects. These are related in terms of cause (Incomplete Mineralization and Microbial Growth) and distinct in terms of means to control or avoid. These effects may be the result of exposure to compounds produced as a result of the treatment process or to microbes used or augmented as a result of deliberate alterations of the environmental characteristics of the site.

Incomplete Mineralization

Physical treatment methods will result in the transfer of material from one medium to another (e.g., water to soil, water to air). Microbial biodegradation will, in theory, result in complete mineralization. However, degradation may not be complete and the intermediate products of microbial metabolism may accumulate (i.e., biotransformation vs. biodegradation). These biotransformation products may be less, more, or as toxic as the beginning material. They

may be less, more, or as mobile as the beginning product. They may be less, more, or as persistent as the beginning product. Differences in mobility and/or persistence will lead to changes in exposure levels that could result in adverse effects. Longer exposure to higher levels of a less toxic material could result in unanticipated expression of toxicity. As indicated above, partial degradation of polyvinyl chloride can result in the accumulation of vinyl chloride, a known human carcinogen. Other examples include the conversion of amines to N-nitrosamines in the presence of nitrites or nitrogen oxides (Ayanba and Alexander 1974, Greene et al. 1981) and the accumulation of chlorobenzoate as a result of partial biodegradation of PCB congeners (Sayler et al. 1988).

If partial degradation (biotransformation) occurs, additional risk assessment issues are raised. One must ascertain the toxicity, mobility, and persistence of the accumulated metabolite. These will determine the potential for adverse effects on the environment, nontarget organisms, and humans. The extent and path of partial degradation will determine the type and quantity of compounds present. Many tests are available to test the harmful effects of specific compounds on biological tissue (Loomis 1978, Paustenbach 1989).

However, prediction of the specific metabolite and its concentration may not be possible. Environmental factors (pH, temperature, moisture content) and the presence of indigenous microbes may greatly affect the extent of degradation. Additionally, tests for individual compounds do not provide information on possible synergistic effects of mixtures of chemicals. Tests are available that attempt to measure the toxicity of complex mixtures of chemicals. (Irvin

1989, Irvin and Akgerman 1987, Jones and Peace 1989).

Microbial Growth

It can be assumed that the specific microbes selected (or engineered) for use in degrading the waste material will have been shown to be innocuous, relative to human and other nontarget animals or insects. However, as indicated above, biotreatment involves the addition of nutrients to support the metabolic activity of the desired microbes. This will not create concern in closed systems: however, in noncontained systems the growth of other microbes normally present may occur, including those pathogenic to humans or other nontarget animals or insects. Exposure of workers or populations to these microbes would result in adverse effects.

Health issues related to incomplete mineralization would result from exposure of populations via either contaminated water or air. If it has been demonstrated that groundwater contamination is possible, water safety can be assured by the use of test wells that permit the monitoring of effluent from the site. Similarly, discharge water can be monitored. It must be stressed that monitoring need be conducted only if the metabolic intermediates are known to be hazardous and there is expectation that incomplete digestion is likely. Airborne contamination, wherein microbes are dispersed generally by dust particles, can be dealt with as described below (Problems Associated with Non-enclosed Methods).

Environmental Issues

There has been much public concern and speculation about the possibility of adverse environmental effects from the environmental application of runaway engineered organisms. The possibility of effects on nontarget organisms, on biological cycles, and on human health has been discussed. To date, after almost 700 field tests of engineered microbes or plants, there is no record of such problems arising. In one instance (Short et al. 1991) researchers evaluating the efficacy of Pseudomonas strains that had been engineered to degrade 2-4 Dichlorophenoxyacetate found that 2-4 Dichlorophenol (a toxic intermediate metabolite) accumulated in the soil. The accumulation of 2-4 Dichlorophenol resulted in a loss of 90% of the fungal population in the soil. The possibility of an adverse effect requires that environmental applications be reviewed for safety considerations.

Cavalieri (1991) has proposed that microcosms be used to predict the environmental consequences of the application of engineered microorganisms. Microcosms can provide information about persistence, survival, and specific effects of the modified microbe in question relative to the unmodified host. While the information from microcosms may not be entirely representative of results under field conditions, it will provide a basis for deciding whether or how field testing should proceed. Similarly, based on microcosm data, the process could be modified, safety precautions instituted or devised, provisions for confinement or mitigation devised, and effective monitoring protocols designed.

Problems Associated with Non-enclosed Methods

There is considerable variation in the methods for biotreatment due to the variable nature of the material to be treated, physical characteristics of the site, climate, and regulatory considerations. Clearly the more control the operator has over the bioremediation system the greater the likelihood of a successful outcome with the least likelihood of adverse effects. The batch reactor provides the most control, followed by the various types of holding tanks or semienclosed bioreactors. Finally, natural or modified ecosystems provide the least control. Batch reactors are closed systems and the microbes can be thought of as contained and not free to enter the environment. At the same time the physical/chemical environment can be controlled to assure complete mineralization. Holding tanks and semienclosed reactors provide limited control. These vary from small to large open (fenced to restrict entry) or covered lagoon type enclosures to greenhouse type structures covering mounds of contaminated soil.

These semienclosed systems may employ either an augmentation process in which additional microbes are added or are treated by adding nutrients to enhance the growth of indigenous microbes. Very often nutrients and microbes are added simultaneously. Most microbes in natural situations are not identifiable. (5% of the microbes in a soil sample cannot be cultured in laboratory situations, generally due to the lack of an appropriate media, and are considered to be in a viable, non-culturable state.) With the addition of nutrients in uncharacterized field situations bacteria, fungi, and protozoans will multiply. Many of these will have their associated viruses. Some of these microbes could be human, animal, or plant pathogens, responding to the added nutrients and altered growth conditions.

These microbes may cause infection, allergic reactions (especially among workers on the site), or produce toxins. Perhaps the best example of a normal soil bacterium that can cause infection is Clostridium tetani, which infects through a puncture wound. Other bacteria, such as Bacillus subtilus are known to produce allergic reactions in workers. According to Emmons (1962) "the fungi that cause systemic mycoses are normal and more or less permanent members of the soil". In addition, exposure to fungi results in allergic reactions and some fungi produce toxins.

The use of enclosed systems (i.e., covering the lagoon or reactor with canvas or plastic) is encouraged to minimize the dispersal of microbes. While enclosing the site will minimize exposure to the general public, within the closed system workers may be exposed to high concentrations of microbes via dust particles or spray. Moistening the surface of the soil being treated at sites will reduce the amount of dust in the air. In some cases face masks may be advisable.

Containment and Mitigation

Total eradication of unwanted microorganisms is rare, but reduction to acceptable levels (i.e., below the level of unacceptable economic or health impact) is possible. Absolute containment of microorganisms is not possible and, based on experience with both beneficial and detrimental microorganisms, not essential (Vidaver and Stotzky 1992). Vidaver and Stotzky propose the use of the more realistic term "confinement" in lieu of containment. Confinement

does not imply that the microbe will not spread beyond the point of application, but rather that it can be effectively managed and adverse effects minimized. Most microorganisms are confined biologically by their individual requirements for nutrients, moisture, and sensitivity to environmental conditions (i.e., their niche).

Additional strategies involve the use of debilitated organisms or the construction and use of safe cloning vectors with limited ability to transfer or survive outside the original host and the use of replicons sensitive to temperature or other environmental factors (Cuskey 1992). The use of debilitated microbes is not practical for environmental applications. However, several conditionally lethal systems for the control of released bacteria have been designed and tested. These include a temperature sensitive system (e.g., where DNA repair does not occur at cold temperatures), a conditionally lethal construct wherein the organism has an inducible metabolic pathway that can be activated only by the presence of an innocuous chemical not normally present in the environment of the microbe and a "suicide" gene that will destroy a key feature without which the cell cannot survive. The gene is controlled by the presence (induced) or absence (derepressed) of the waste in question. If the waste concentration falls below a critical level, the gene is activated. Alternatively the gene is always active and a second gene provides protection. Activity of the second gene is controlled by the concentration of the waste being treated.

Decontamination (or mitigation) of the environment of microbes has been studied and is discussed by Vidaver and Stotzky (1992). It is important to keep in mind that each situation is

different and that procedures for decontamination will differ. A case by case approach is essential. The type of organism, the physical environment, the nature of the modification and the season must all be considered. Knowledge about the organism, whether it is a wild type or has been modified, is critical to designing a decontam-ination protocol. Table Seven lists methods of decontaminating soils, plants, and animals, if they are contaminated with a hazardous microbe, and provides an indication of the time required to achieve effectiveness. Plants and animals are presented because of the possibility of contamination during a field application. Thus, in the event of contamination of animals (straying on the site) incineration, quarantine, or slaughter could be employed immediately to minimize spread of the microorganism. Birds, rodents, and runoff water must be considered as alternate sources of microbial dispersal. Plants that are growing on the site may be contaminated with the microbe. If the microbe is considered a hazard the plants should be immediately destroyed, (i.e., burning, tillage) or quarantined if a future use is being considered. Long term solutions are presented for use in the event the project has a long life span and the problem is recurrent. The issue of physical security, especially with animals, cannot be overstressed. Strong, tall fences will eliminate the presence of most unwanted mammals and insure against trespassers.

Details of soil sterilization to decrease the bacterial levels at the site are given in Table Eight. Specific soil fumigants in common use in the US are identified. As can be seen, most are general in effectiveness. All are toxic to plants and animals and must be used with care. Use of a fumigant will significantly lower the densities of all microorganisms present in the soil. Steril-ization is not achieved. As a result, over time, the remnants of the microbial flora will

reproduce and cell densities will increase. The new population may be similar to the previous one, in terms of types and relative numbers of individual types or it may differ radically, depending on which portion of the population survived the fumigation and at what level. There is a possibility that the introduced microbe could be the dominant type. For this reason, it is recommended that the treated site be reinoculated with uncontaminated soil from the surrounding area. This will most likely result in replacement of the original indigenous microbial flora and significantly decrease the probability that the introduced microbe would flourish.

Lamptey et al. (1992) discuss methods of decontamination specifically oriented toward small or large scale field trials with Bacillus, which are generally more refractive. They suggest that if the problem area is small enough the upper layer of soil (including plants and associated fauna) could be dug up and sterilized. They suggest the use of steam (121°C/15 min) or irradiation (^{60}C source, 3000 Krad/H for 3H or 3Krad/H for 96H). For larger sites, where excavation would be impractical, direct application of steam is recommended. This can be accomplished by burying steam pipes (80cm apart) and supplying steam from a boiler ($1x10^6$ Kcal/H). A more widely used system is "steam stripping" wherein PVC sheets are spread over the area to be treated, weighted down and steam is pumped under the sheets. Temperatures ranging from 54-100°C have been observed. The process can be repeated at intervals to destroy germinated spores. These procedures could be used with any microorganism.

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Table One: Sample of Waste Types at a Superfund NPL Site

Material	Quantity (gallons)
Oyster shells with copper	6000
Oil and water	58,150
Paint	2,457,904
Perchloroethylene	800
Paint/Formaldehyde	4250
Paint thinner/stripper	90,025
Paint and plastic sludge	251,885
Polychlorinated biphenyls	14,000
Paint sludge and epoxy	9,740 yd(3)
Pesticide-affected fabric	500 (lbs)
Perchlorethylene, oil, and alcohol	18,400
Pesticides	7,582
Phenolic resins	89,360
Phenol-formic acid and methylene	900
Phosphoric acid solution	2,940
Phosphorus	350 (lbs)
Potassium cyanide and candy	168 (lbs)
Poisoned cookies, arsenic (box)	2

Table Two: Nutritional Versatility of Strains When Selected Hyrdocarbons are Present as the Sole Carbon and Energy Source

	P. putida	P. oleovorans	6Dp	Dl	PB
Toluene	+b	+	+++	-	+
2-Cl-toluene	++	++	+++	++	+
3-Cl-toluene	+++	+++	++++	+++	+++
3,4-diCl-toluene	+++	+++	++++	+++	+++
2,6-diCl-toluene	++	++	+++	++	+
Xylenes	+	+	++	+	+
Benzoate	++++	++++	++++	++++	++++
3-Cl-benzoate	-	-	++	-	-
4-Cl-benzoate	+	+	+++	+	+
2,4-diCl-benzoate	++	+	+++	++	+
3,4-diCl-benzoate	++++	++++	++++	+++	++++
2,4-D	++	+++	++++	++	++
2,4-diCl-phenol	+	+	++	+	-
2,4,5-T	++	++	++++	+++	+++

Table Three: Degradation by Microbial Consortia

Degradative Activity	Microorganisms	Reference
Degradation of DDT: p-chloro-phenyl acetic acid produced and then utilized by Arthobacter spp. (Cometabolism)	Hydrogenomas spp and Arthobacter spp.	Pfaender and Alexander
Degradation of Polyvinyl Alcohol: Degradation by Pseudomonas putida provided growth factors for cometabolism to occur. (Cometabolism)	Pseudomonas putida and other Pseudomonas species	Sakazawa et al.
Degradation of Kepone (Cometabolism)	Pseudomonas aeruginosa	Orndorff and Colwell
Degradation of Silvex: pair of microbes grew using Silvex, no growth when separated. (Cometabolism)	Pseudomonas and Achromobacter spp.	Ou and Sikka
Consortia but not pure cultures were able to degrade Trichloro-ethylene	Aerobic degradation yielding HCl and CO_2	Fliermans et al.
Demonstrated "concurrent metabolism" of xenobiotics (present at environmental concentrations) by resting cells. (Consortia)	Wood pulp wastes degraded by stable consortia anaerobically	Nielson et al.

Table Four: Types of Biotreatment Processes

Type	Principle	Primary Application
Sequencing Batch Reactor	Microbial digestion in liquid suspension	Control of reaction conditions; release of microbes to environment
Aqueous Treatment System	Immobilized microbes or enzymes in flow through system	Requires Soluble Organic material. No microbial release
Soil Treatment System	Wash procedure to solubilize adsorbed contaminants	Necessary pretreatment to maximize efficacy
Fixed Film Bioreactor	Microbes/enzymes on plastic media in column to maximize surface area and nutrient exchange	Can treat low concentrations of organic material
Soil Slurry (Tank or Lagoon)	Soil and water agitated together in reactor	No temperature control
Land Farming	Soil mixed with nutrients and tilled in situ	Requires lining to contain microbes and material
Subsurface Reclamation	Water, nutrients, and oxygen (electron acceptor) pumped through soil	Enhanced growth of entire indigenous population. Oil and gasoline spills; organic contamination of ground-water

Table Five: Parameters and Compounds Monitored

For Regulatory Compliance[1]	For Monitoring The Process
pH	Chlorendic Acid
Phenol	Phenol
TOC	Benzoate
Trichloroethylene	o,m,and p Chlorobenzoic Acids
Tetrachloroethylene	pH
Monochlorobenzene	Biological Oxygen Demand
Monochlorotuoluene	Suspended Solids
Benzene	Oxygen Consumption Rate
Trichlorobenzenes	Total Organic Halide
Tetrachlorobenzenes	
Monochlorobenzotrifluoride	
Hexachlorocyclobutadiene	
Hexachlorocyclopentadiene	
2,3,5-Trichlorophenol	

[1]Must meet compliance levels set by USEPA.

Table Six: Comparison of Treatment Methods

Type of Treatment	Cost ($) per cubic yard	Time Months	Major Problems
Incineration	250-800	6-9	Emissions, Energy
Fixation	90-125	6-9	Decomposition, Leaching
Landfill	150-250	6-9	Seepage, Long term Containment
Biotreatment	40-100	18-60	Metabolic by-products, Time factor, Release of microbes

Table Seven: Time Frames and Methods for Controlling or Eliminating Unwanted Effects of Free-Living Microorganisms Associated with Plants and Animals

Microorganism Association	Immediate[a]	Short-term[b]	Long-term[c]
Free-living	Fumigation Flooding Chemicals[d]	Fumigation Flooding Chemicals Erosion control Soil amendments	Fumigation Flooding Erosion control Soil amendments
Plants	Burning (eradication) Quarantine Tillage Chemicals Irrigation/flooding Insect vector control Machinery sanitation Runoff water control Solarization	Quarantine Chemicals Crop rotation Cultivar rotation Irrigation/flooding Heat treatment Soil solarization Erosion control	Crop rotation Cultivar rotation Soil amendments Weed control Erosion control
Animals	Incineration Quarantine Slaughter Bird, rodent, insect control Runoff water control (insects) Physical security	Quarantine Antibiotics, drugs Bird, rodent, insect control Physical security	Anitbiotics, drugs Bird, rodent, insect control Physical security

[a]Hours to several days to achieve effectiveness.
[b]0-3 years to achieve effectiveness.
[c]Longer than 3 years.
[d]Choice and availability of chemical for target microorganisms dictate feasibility and approach.
Adapted from Vidaver and Stoztky, 1992.

Table Eight: Soil Fumigants

Common name	Chemical name (Some trade names)	Formulation	Specificity	Dosage, Amt/ha	Toxicities: Plant	Toxicities: Mammalian LD_{50a}	Application considerations
Methyl bromide	Bromomethane (Dowfume MC-2)	98%+2% chloropicrin	General biocide	450-900 kg	Toxic	1 mg/kg	Requires gas-proof seal
Chloropicrin	Trichloronitromethane (Picfume, Larvacide)	100%	General biocide	300-500 liters	Toxic	1 mg/kg	Best activity with gas proof seal
Chlorinated hydrocarbons (1,3D)(DD)	1,2-Dichloropropane, 1,3-dichioropropene, & other chlorinated hydrocarbons, (Telone, Vidden D)	1,3-D alone or with other chlorinated hydrocarbons	Nematicidal	100-500 liters	Toxic	140 mg/kg	Requires soil seal
Ethylene dibromide (EDB)	1,2-Dibromoethane (Dowfume W-84, Nematox 100)	60-85% in liquid	Nematicidal	19-94 liters	Toxic	150 mg/kg	Requires soil seal
Methyl isothiocyanide	Methyl isothiocyanid is added directly or is the active breakdown product of several unstable compounds	30-40% liquid or wettable powder	General biocide	600-1200 liters or 300-400 kg	Toxic	280-650 mg/kg	Injected or rotovated in
Dibromochloropropane[b] (DBCP)	1,2-Dibromo-3-chloropropane (Fumazone, Nemagon, etc.)	Liquid	Nematicidal	19-38 liters	Toxic to some plants	172 mg/kg	Injected or drenched
Hypochlorite	Chlorine	100 ppm in water	Microbiocine	PH & temp dependent	Toxic	.03-.2[c] mg/kg	Applied as liquid

[a]LD_{50} is the dosage lethal to 50 percent of a test (usually rat) population.
[b]Because of toxicities, DBCP is no longer used. It is included here for comparisons only.
[c]Sensitivity range for continuous exposure of sensitive fish species: Le_{50} (Lethal concentration for 50%).
Adapted from Vidaver and Stoztky (1992).

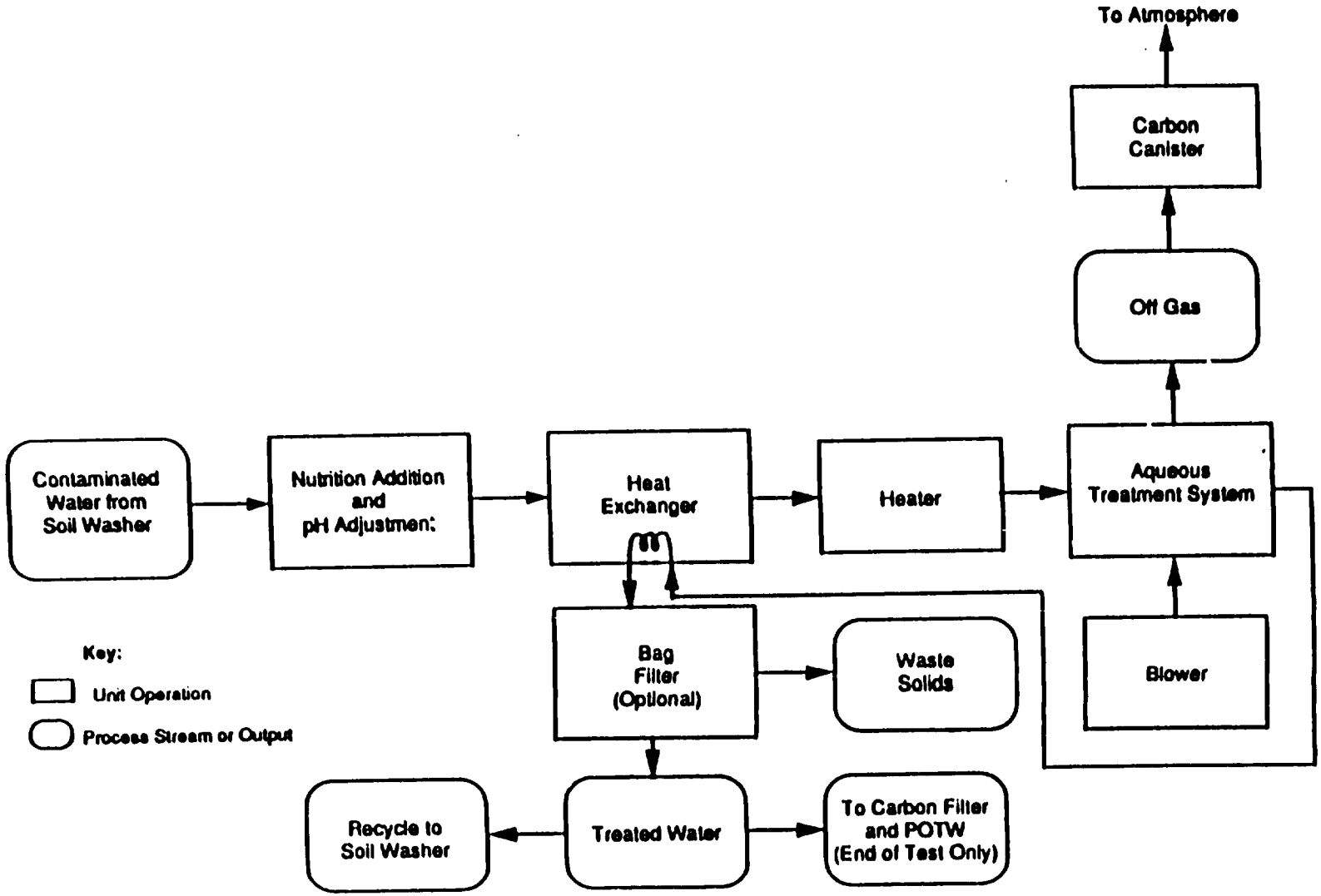

Figure 1 Flow Diagram of Aqueous Treatment Systems (ATS)
(Ellis and Stinson 1991)