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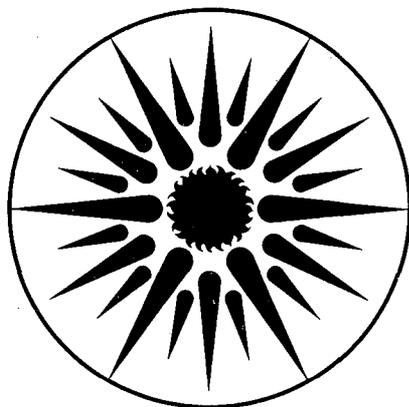
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ISSUES RELEVANT TO BIODEGRADATION OF ENERGY-RELATED
COMPOUNDS IN GROUND WATER: A Literature Review

J.B. Healy, Jr. and C.G. Daughton

September 1986

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**ISSUES RELEVANT TO BIODEGRADATION OF
ENERGY-RELATED COMPOUNDS IN GROUND WATER**
a Literature Review

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ABSTRACT

Organic compounds released to the environment by hydrocarbon extraction/conversion processes (i.e., synfuel and enhanced oil recovery processes) could be major contributors to the growing problem of groundwater contamination. This could be a major problem for a fully developed commercial-scale synfuels industry. One of the least understood, but potentially most important, processes affecting groundwater quality is subsurface biodegradation of chemical contaminants. This report summarizes the current state of knowledge on in-situ biodegradation of groundwater contaminants, points out the limitations of current research, and makes recommendations for future research. The sparse literature on the fates of organic contaminants in the subsurface environment provides few, well-documented descriptions of in-situ transformations of organic compounds by indigenous microbiota. In-situ restoration of contaminated aquifers through enhancement of naturally occurring biodegradation processes, however, shows definite promise as an economical means of removing contaminants from the groundwater environment. In contrast, the effectiveness of purposeful introduction of specially adapted or genetically engineered microorganisms remains purely conjectural. To obtain a better understanding of the processes controlling the in-situ fates of organic contaminants, future research should emphasize definitive experimental controls, reliable and extensive sampling of both the microbiota and the parent contaminant or metabolites, increased authenticity in laboratory simulation experiments, and integration of concomitant laboratory and field studies. Only after a dramatic increase in funding to implement these recommendations, can the research community expect to make reliable predictions of the fates of groundwater contaminants and design appropriate aquifer-restoration schemes.

CONTENTS

| | |
|--|-----------|
| Abstract | ii |
| I. Introduction | 1 |
| Objectives | 3 |
| II. In-Situ Biodegradation of Compounds from Hydrocarbon/Extraction Conversion Processes (HECP) | 3 |
| BEST AVAILABLE DOCUMENTATION | 4 |
| Existence of microbes in the subsurface | 4 |
| Morphology and diversity of subsurface microbes | 5 |
| In-situ microbial activity | 6 |
| Issues in groundwater metabolism | 8 |
| WEAKNESSES IN CLAIMS OF IN-SITU BIODEGRADATION | 10 |
| Mass balances | 11 |
| Circumstantial evidence | 11 |
| unique biological responses | 11 |
| cell-number estimation and sampling | 13 |
| spatial-sampling evidence | 15 |
| aquifer restoration | 15 |
| Abiotic controls | 18 |
| dispersion | 18 |
| sorption | 19 |
| volatilization | 20 |
| chemical transformation | 20 |
| Sampling controls | 21 |
| Laboratory-scale simulations | 21 |
| III. Conclusions | 25 |
| IV. Recommendations for Future Research | 25 |
| V. Acknowledgments | 28 |
| VI. References Cited | 29 |
| Table I. | 41 |
| Most Abundant or Frequently Occurring Compounds from the Major Chemical Classes Occurring in Oil Shale Process Streams | |

I. INTRODUCTION

Ground water is a critical resource in the United States because half of the population relies on subsurface aquifers for drinking water, and many agricultural and industrial operations require high-quality ground water (Bitton and Gerba 1984; Ward, Giger, and McCarty 1985). Once thought to be a rare occurrence, widespread contamination of ground water has only recently come to the attention of citizens, industry, and government agencies. Although the vast majority of aquifers in this country (comprising most of our groundwater resource) remain in their native, pristine states (Lehr 1985; Sun 1986), many of these are at risk with the advent of large-scale commercial synfuel developments, especially those in unpopulated areas.

Ground water can be contaminated by numerous organic chemicals used by or produced during hydrocarbon extraction/conversion processes (HECP) (e.g., synfuel and enhanced oil production). Contamination of ground water by these processes can result from (i) leaching of compounds endogenous to native, undisturbed oil shale, coal, tar sands, and petroleum deposits (physical alteration of the deposit structure by blasting and mining exacerbates this problem), (ii) pyrolysis/combustion products from in-situ processing of oil shale, coal, and tar sands, (iii) pyrolysis/combustion products from ex-situ (above-ground) processes, which subsequently are transported to surface/subsurface waters during and after waste storage or disposal, (iv) fugitive atmospheric emissions and photochemical transformation products that are subsequently transported to the surface by precipitation, and (v) compounds purposefully introduced during mining/extraction/conversion to facilitate or enhance product recovery (e.g., explosives during mining; surfactants used for enhanced oil recovery). Examples of these processes include oil shale conversion (Daughton 1986; Leenheer and Stuber 1981; Riley et al. 1981), disposal of waste residues from a coal-gas plant (Burnham et al. 1972, 1973), natural gas and oil production (Eiceman et al. 1986), and underground coal gasification (Humenick, Britton, and Mattox 1982; Mattox and Humenick 1980; Stuermer, Ng, and Morris 1982). As evident from a general overview of groundwater quality that was recently published (Ward et al. 1985), it is of critical importance that we increase our understanding of all processes affecting groundwater quality so that we can maintain uncontaminated groundwater supplies for present and future needs in addition to upgrading already contaminated aquifers.

The ultimate fate of compounds in the groundwater environment is governed by numerous transport and transformation phenomena. The former tend to be abiotic processes and include numerous modes of sorption (e.g., chemisorption and organic-phase absorption/solvation), dispersion, convection, and volatilization to the pore structure of the vadose zone; some of these simply serve to immobilize the compound, making it unavailable to other environmental compartments. The latter include chemical alteration (e.g., surface-catalyzed hydrolysis, ligand formation/precipitation, and oxidation reduction) and numerous modes of bioalteration.

Potentially the most important, but currently the least understood, process affecting groundwater quality is subsurface biodegradation of chemical contaminants by indigenous microorganisms. Renewed interest in subsurface biological activity was established with the appearance of a 1975 literature review that discussed factors likely to be important to subsurface biological activity (McNabb and Dunlap 1975). Most of the important aspects of groundwater microbiology that are currently known are covered in three recent reviews (Bitton and Gerba 1984; Hutchins et al. 1985; Ward et al. 1985).

Naturally occurring biodegradation could play a major role in determining the fate of aquifer contaminants. More importantly, however, microbial activity has the potential to be manipulated to enhance this decontamination process. Restoration of contaminated aquifers by enhancement of in-situ biodegradation (bioreclamation) could be the most cost effective and least disruptive treatment option in the future (Flathman and Githens 1985; Lee and Ward 1985). Advantages and disadvantages of this approach have been discussed (Lee and Ward 1985). An extension of this approach, purposeful inoculation (seeding) with specifically adapted or genetically engineered microorganisms, is in a pioneering stage of development. This latter approach was an early consideration for removal of petroleum from spills and for emulsification of petroleum residues in ship ballasts (Atlas and Bartha 1973). For other environmental contaminants (esp. pesticides and other EPA Priority Pollutants), it has mainly been investigated for surface waters and soil but only on a small scale (Goldstein, Mallory, and Alexander 1985). With respect to this very limited state of knowledge, it is important to understand the role of indigenous, in-situ biodegradation and to gain experience in manipulating the groundwater environment to obtain better predictions of the fates of organic contaminants.

Objectives

The objectives of this report were to critically review the literature on groundwater microbiology and to make recommendations to the Department of Energy on future research needs regarding microbial degradation of HECF contaminants in ground water. We cannot overemphasize that in the sense that this is a critical review, we have in no way intended to discredit the many competent researchers who have been and continue to pioneer in this extremely difficult field. Any criticisms are merely the inevitable reflection of the enormous complexity of doing groundwater research, especially that done under field conditions.

The reviewed literature mainly encompassed refereed and archival journals and books (i.e., the open literature). Most of the "gray" literature, which in many respects can be extensive (e.g., corporate/government reports), was avoided because of dubious scientific merit or lack of wide availability; both the EPA (Lobel 1986) and DOE (Wobber 1985) have active research programs in the area of subsurface transport and transformation of organic compounds. Much of the gray literature concerns the pervasive national problem of groundwater pollution by gasoline, other petroleum products, and hazardous wastes and is not related to problems unique to synfuels. This report describes the current state of knowledge on in-situ biodegradation of groundwater contaminants, summarizes the limitations of current research, and makes recommendations for future research.

II. IN-SITU BIODEGRADATION OF COMPOUNDS FROM HYDROCARBON EXTRACTION/CONVERSION PROCESSES (HECF)

The compounds resulting from HECF waste storage/disposal belong to numerous chemical classes (Table I). Nearly all of the extensive literature on biodegradation of compounds from these classes may not be directly relevant to what may occur in ground water. This aspect will not be discussed, but literature reviews on the biodegradation of some of these classes include: aromatic compounds in anaerobic (Evans 1977; Young 1984) and aerobic (Alexander and Lustigman 1966; Evans 1963) environments and heterocycles (Callely 1978; Naik et al. 1972; Sims and Sommers 1986). Representatives of many of these classes are considered resistant to biodegradation and are covered in a general review (Ludzack and Ettinger 1960). Specific reports, concerning the biodegradability of synfuel wastewaters or major constituents in these wastewaters include: coal gasification (Luthy, Stamoudis, and Campbell 1983; Stamoudis and Luthy 1980), oil shale (Dobson et al. 1985;

Healy, Jr., Langlois, and Daughton 1985; Rogers et al. 1981, 1985), and tar sands (Torpy, Luthy, and Raphaelian 1983).

The fate of HECPC compounds in a particular groundwater environment will depend on the specific hydrogeology and groundwater flow characteristics, the particular physicochemical environment controlling sorption and chemical transformations, and the presence of microorganisms and biologically favorable conditions. The relative significance of these interactive characteristics in determining the fate of contaminants can differ for any given compound, groundwater location, or sample site. The ability to distinguish the in-situ significance of individual transformation or immobilization processes is currently impossible in the groundwater environment because there is no way to control a sufficient number of variables without simultaneously altering the environment being measured. Some of these problems will be overcome with the further development and application of remote-sensing methods of chemical analysis (Fitch and Gargus 1985; Hirschfeld et al. 1984; Milanovich 1986). Because groundwater microbiology is a relatively new science with few guidelines or agreed-upon criteria for qualitatively or quantitatively demonstrating in-situ biodegradation, it is extremely important to use caution when evaluating claims of groundwater biodegradation.

Best Available Documentation

Existence of microbes in the subsurface. Only recently has the subsurface environment been implicated as having an indigenous microbiota. Five reports, however, clearly demonstrate the existence of microbes in the subsurface (Ghiorse and Balkwill 1983; Lee, Wilson, and Ward 1984; Ward 1985; Whitelaw and Rees 1980; Wilson et al. 1983). Although the types of in-situ metabolism were not established in these reports, both aerobic and anaerobic (denitrification) respiration have been demonstrated in laboratory studies for subsurface isolates (Ward 1985; Whitelaw and Rees 1980). Even though eucaryotes were not found in carefully sampled core material (Ghiorse and Balkwill 1983; Wilson et al. 1983), fungi and protozoa have been identified in well-water samples (Hirsch and Rades-Rohkohl 1983). All other reports of the occurrence of microbes in the subsurface environment have not appeared to have eliminated the possibility of sample contamination by surface microorganisms or those associated with the immediate sampling-well environment. This is a major problem with most studies.

Only two reports (Lee et al. 1984; Webster et al. 1985) clearly demonstrate that viable microorganisms exist in a subsurface environment and are capable of degrading HECF contaminants. This evidence was obtainable because a sterile core-sampling technique was used (Wilson et al. 1983). Both attached and free-living bacteria present in the sample at the time of collection were counted by eye with epifluorescent microscopy and an acridine orange staining technique (Ghiorse and Balkwill 1983). In addition, viable-cell counts were estimated from growth on solid media.

An important question is whether the bacterial populations found in the subsurface (Lee et al. 1984; Webster et al. 1985) are indigenous (autochthonous) and have existed prior to the arrival of the contaminant plume. Stimulation of an indigenous population by nutrient addition to a contaminated aquifer has been attempted for aquifer restoration (Jamison, Raymond, and Hudson, Jr. 1975). It is possible that some or all of the subsurface bacteria could have migrated from the surface with the contaminant plume and therefore should be considered as exotic (foreign or allochthonous) bacteria. Regardless of their origin, a further question is whether these bacteria are capable of biodegradation only under surface or laboratory conditions and exist functionless in the subsurface.

An important issue in subsurface microbiology is whether bacteria can be widely dispersed by a contaminant plume or by purposeful injection. Microbial contaminants have been reported to have been transported great distances in certain aquifers (Gerba 1985; Zoeteman 1985) although cracks and fissures may have channeled the transport of these bacteria (Wood and Ehrlich 1978) while their wide dispersal was probably limited by the filtering ability of solid subsurface material. Indeed, it is generally believed that most compacted soil types filter out bacteria-sized particles (Crane and Moore 1984). Resolution of the issue of bacterial transport in the subsurface will have critical implications on proposed aquifer restoration schemes that involve purposeful inoculation with specially adapted microorganisms because these methods would rely on wide dispersal of the inoculum for maximum exposure to chemical contaminants.

Morphology and diversity of subsurface microbes. Subsurface bacteria obtained from aseptically sampled core material from uncontaminated shallow aquifers have been observed with epifluorescent microscopy (acridine-orange stained cells) and transmission electron microscopy (TEM) (Balkwill and Ghiorse 1985; Ghiorse and Balkwill 1983; Wilson et al. 1983). Cell morphologies were diverse, and a greater proportion of Gram-positive than Gram-negative bacteria was observed. Most of the cells were coccoid and small

(<1 μm diam.). Many of the bacteria lacked cytoplasmic constituents and contained polyhydroxybutyrate (PHB)-like storage inclusions suggesting that they were adapted to oligotrophic environments (Poindexter 1981). Some of the bacteria were associated in a glycocalyx-like material implying attachment to surfaces (e.g., biofilm). A significant fraction of subsurface bacteria appears to be attached to surfaces (Harvey, Smith, and George 1984; Ventullo, Ladd, and Costerton 1983), although the efficiency of detachment by methods used to remove organisms from surfaces before counting has not been reported. Some bacteria observed by TEM contain internal membrane systems typical of nitrifying or methane-oxidizing bacteria. It appears that samples from two shallow aquifers (Balkwill and Ghiorse 1985) contained a predominance of aerobic, nutritionally versatile bacteria that were active in an oligotrophic subsurface environment without forming dormant cell structures. In general, however, no statement can be made regarding a predominant form of metabolism in the subsurface, although it is reasonable to assume that most metabolism is mediated by attached organisms.

In-situ microbial activity. Ideally, biodegradation is documented by demonstrating at least one of the following: (i) reduction of substrate concentration coupled with an increase in biomass concentration (assuming the absence of various sorption processes), (ii) production of new compounds that are unique and direct representative products of biodegradation (e.g., catabolites), or (iii) production of compounds that result indirectly from catabolism (e.g., reduction of terminal electron acceptors) or onset of metabolic behavior indicative of catabolism (e.g., acclimation of microbial population or lag-time reduction for substrate attack).

A number of studies have demonstrated that in-situ biodegradation may have occurred at sites where ground water had been contaminated with compounds common to HECF wastes. The most convincing evidence for in-situ activity has resulted from the detection of methane gas, an end product of anaerobic metabolism, in two different aquifers contaminated by creosote wastes (a mixture of phenolic and polynuclear aromatic/heteroaromatic hydrocarbons) (Ehrlich et al. 1982, 1983; Godsy, Troutman, and Ehrlich 1983; Goerlitz et al. 1985). Methane could not be detected from uncontaminated portions of these aquifers. Because the surface-generated waste did not already contain methane and because abiotic formation of methane in these contaminated aquifers was presumably impossible, it appears that the methane was produced by the microbial conversion of some of the organic contaminants. The possibility that the detected methane originated at the surface prior to the entry of the waste plume into the ground water was

ruled out by the methane concentration profile in the subsurface. The methane concentration increased with increasing waste-migration distance from the point of entry at the surface (unlined surface impoundments and contaminated wetlands). If methane were in the waste prior to contamination of the ground water, the concentration would have decreased as the waste plume migrated through the aquifer because of dispersion and volatilization and, in addition, perhaps biooxidation.

Migration of creosote wastes in a sand-and-gravel aquifer that had been contaminated from unlined surface impoundments at Pensacola, Florida, has been investigated (Goerlitz et al. 1985). Sorption did not influence the retardation of naphthalene and substituted phenols during migration. That only certain aromatic compounds disappeared while others remained supports the occurrence of biodegradation because bacteria preferred certain chemical classes and isomers over others (Goerlitz et al. 1985). Loss or reduction in concentration caused by abiotic mechanisms would be less selective within a given chemical class. One of these aromatic compounds, a xylenol isomer (3,5-dimethylphenol), that did not disappear (presumably because it resisted biodegradation) was used as a tracer to model the contribution to reduction in substituted-phenol concentration solely as a result of dispersion during migration of the waste plume. A series of monitoring wells positioned along a gradient line of increasing distance from the surface impoundments were used to monitor the waste migration. Evidence that methane was being formed in-situ was provided by the fluctuating methane concentrations (all significantly above ambient levels) along the dispersion gradient from the impoundment as detected in the series of multi-level monitoring wells. This methane-concentration profile rules out the possibility that methane was merely transported from the surface impoundment because dispersion would have caused a decreasing concentration profile along the migration route. The geologic formation did not produce a significant amount of methane, and short-term, abiotic mechanisms for in-situ formation of methane are not known. Therefore, in-situ microbial formation of the methane was the only reasonable explanation for the presence of methane in the contaminated aquifer. Some of the substituted phenols that disappeared in the aquifer were shown to be methanogenic substrates in laboratory experiments (Ehrlich et al. 1982) and presumably supported some of the in-situ methanogenesis.

Another example of the detection of a unique metabolic end product for the demonstration of in-situ microbial activity was described for an uncontaminated aquifer that presumably had never been significantly polluted by human activity and contained only naturally occurring organic matter (Vogel, Talma, and Heaton 1981). This study

detected an onset of molecular nitrogen production only after oxygen was depleted. The nitrate concentration decreased as the molecular nitrogen concentration increased. These data were explained by the fact that oxygen is consumed as an electron acceptor before nitrate is converted to nitrogen gas in the anaerobic process of denitrification. Further evidence for the biological origin of the molecular nitrogen was provided by isotope analysis of the biological partitioning of ^{15}N and ^{14}N . Other potential sources of nitrogen were ruled out.

In addition to the above reports that detected methane and methanogens only in contaminated wells (Ehrlich et al. 1982; Goerlitz et al. 1985), three other reports contained evidence suggestive that bacteria had acclimated to groundwater contaminants in situ. The microbes in ground water sampled from an aquifer contaminated with coal-tar products were better acclimated to the degradation of model contaminant aromatic compounds than microbes in samples from uncontaminated sites (Lee et al. 1984; Ogawa, Junk, and Svec 1981; Wilson et al. 1985). These latter three studies, however, did not show conclusively that in-situ biodegradation had occurred with a concomitant increase in number of adapted microorganisms.

Issues in groundwater metabolism. It is possible that subsurface isolates grown in the laboratory under nutrient-rich conditions or at high substrate concentrations (500 mg/L) (e.g., Lee et al. 1984) do not represent the predominant organisms active in the oligotrophic (nutrient-poor) subsurface. It is generally accepted that oligotrophic bacteria should be studied at the same trace-nutrient concentrations that exist in their native environments because studies using high substrate concentrations do not necessarily predict biodegradation rates at trace concentrations (Boethling and Alexander 1979a; Rubin, Subba-Rao, and Alexander 1982; Russell and Baldwin 1979a). For example, greater numbers of groundwater microbes have been found to grow on nutrient-poor than on nutrient-rich pour-plates (Balkwill and Ghiorse 1985).

Growth on and biodegradation of trace concentrations of organic compounds is a relatively new area of research that is very important to the question of whether indigenous or exotic bacteria will be able to biodegrade trace contaminants in the oligotrophic subsurface environment. A number of recent studies have begun to investigate the ability of bacteria to grow on substrates present at very low concentrations (Boethling and Alexander 1979a, 1979b; Larson and Davidson 1982; Rubin and Alexander 1983; Rubin et al. 1982; Spain and Van Veld 1983; Subba-Rao and Alexander 1982; Subba-Rao, Rubin,

and Alexander 1982). Some studies have specifically addressed bacterial growth on trace organic compounds in subsurface environments (Bouwer and McCarty 1984; Bouwer, McCarty, and Lance 1984; Rittmann, McCarty, and Roberts 1980).

The issue of oligotrophic adaptation and growth is only relevant for compounds dissolved in the aqueous phase. A separate research area is the metabolism of compounds sorbed to solid surfaces (Li and DiGiano 1983; Marshall 1985) and metabolism at the interface of immiscible phases. This problem could be relevant to the subsurface environment where nonpolar, water-insoluble compounds may accumulate at high concentrations (Rostad, Pereira, and Hult 1985). For this situation, organic carbon may no longer be the limiting nutrient. Sources of phosphorus, nitrogen, or sulfur may become more important. Direct (extracellular) metabolism of a sorbed substrate is difficult to prove, and it seems more likely that biodegradation of sorbed substrates involves desorption prior to uptake across the cell membrane (Bouwer and McCarty 1982).

The rate of any biodegradation process is directly related to the size of the bacterial populations and the effective substrate concentrations (Boethling and Alexander 1979a; Simpkins and Alexander 1984; Spain and Van Veld 1983). Before a functional population of bacteria can exist in the subsurface, acclimation must occur. Pre-adaptation or prior exposure of bacteria can often dramatically decrease the time required for biodegradation of contaminants (Spain, Pritchard, and Bourquin 1980; Spain and Van Veld 1983) by reducing the acclimation lag time. The acclimation lag is often necessary for slow mutations to be expressed in a given population before the ability to degrade new materials can effectively take place. Some current evidence indicates that even with lengthy exposure (e.g., millennia) to naturally occurring energy-related contaminants such as alkylpyridines (Riley et al. 1981) or resorcinol (Anonymous 1986; Jolley et al. 1986), these compounds persist. If present, the subsurface bacteria and their associated environments appear unable to effectively biodegrade these contaminants. This is most likely a result of trace-nutrient limitations. Alternatively, suitable microorganisms might not exist in these particular groundwater environments.

Acclimation appears to require a minimum (threshold) concentration for many contaminants, especially those that are not usual substrates (e.g., xenobiotics) (Spain and Van Veld 1983). Organic compounds present below the acclimation threshold concentration would not be biodegraded because a population of adapted microorganisms of sufficient size could not develop.

Although instances of biodegradation of trace organic contaminants that might not be supporting growth have been reported (Bouwer and McCarty 1984; McCarty, Reinhard, and Rittmann 1981; Rittmann et al. 1980; Schmidt, Simkins, and Alexander 1985), it appears that most trace organic compounds will support growth unless they are below specific growth-threshold concentrations (Jannasch 1967; Law and Button 1977; Subba-Rao et al. 1982). Lack of biodegradation could result from an insufficient supply of energy and carbon necessary for maintenance of basal metabolic activities within the cell (Boethling and Alexander 1979b; Pirt 1965; Russell and Baldwin 1979a, 1979b; Van Der Kooij, Visser, and Oranje 1982). Even more energy and carbon would be required for significant cell growth and reproduction, a process often required for acclimation. In some instances, thresholds can be lowered by the presence of additional trace nutrients or organic substrates (Bouwer and McCarty 1984; Hutchins et al. 1984a; Law and Button 1977; McCarty et al. 1981; Rittmann et al. 1980). Even if the same population can simultaneously degrade at least several different substrates at trace concentrations, the growth rate may still be too low to support rapid biodegradation (Van Der Kooij, Oranje, and Hijnen 1982).

A related area of research in which little has been published involves the study of microbial metabolism of complex mixtures (different chemical classes or homologous series) of organic compounds, when each member is present at a trace concentration (Harder and Dijkhuizen 1982; Van Der Kooij, Oranje, and Hijnen 1982; Van Der Kooij, Visser, and Oranje 1982). There are some indications of preferential substrate metabolism (Daughton, Cook, and Alexander 1979; Kompala et al. 1986; Russell and Baldwin 1978; Schmidt and Alexander 1985) and that matrix effects of the mixture can affect the rate of biodegradation (Rubin and Alexander 1983; Russell, Delfino, and Baldwin 1979). There are also reports of trace organic solutes that appear to be more refractory when present in complex mixtures than in individual solutions (Healy, Jr. et al. 1985; Riley et al. 1981).

Weaknesses in Claims of In-Situ Biodegradation

Biodegradation is most easily documented under carefully controlled laboratory conditions allowing for conclusive mass balances. Because of the complexity of subsurface environments and the inability to run controlled experiments in situ, most investigations lack conclusive evidence for in-situ biodegradation. Claims of in-situ biodegradation are often just speculation based upon circumstantial evidence and assumptions (e.g., Flathman and Githens 1985; Jamison et al. 1975; McKee, Laverty, and Hertel 1972; NWWA 1984; Werner 1985). Many of the assumptions are imposed by sampling constraints or low

authenticity in laboratory-scale simulation experiments. For proper perspective, it is important to keep in mind that most of the problems and shortcomings of groundwater microbiology reflect more general problems that have faced microbial ecologists for many years, and many of these problems are greatly magnified by the complexity of field studies.

Mass balances. Microbial conversion of an organic substrate to ultimate degradation products and cell mass can be easily followed in a controlled, closed environment that allows for mass-balance closures. Rigorous mass-balance closures can be obtained with the use of radiolabelled substrates. Ultimate distribution of metabolites can then be quantified for both the abiotic and biotic compartments. But even this method would have shortcomings if it were applied in a field situation because it is theoretically impossible to isolate a portion of the subsurface environment without affecting the very processes that control in-situ biodegradation. In addition, the introduction of radiolabelled materials to ground water is currently prohibited by the Nuclear Regulatory Commission and by individual state agencies. It is unlikely that groundwater microbiologists will ever be able to document in-situ biodegradation of a groundwater contaminant with complete mass closure.

Circumstantial evidence. Without mass balances, the next level of evidence for in-situ biodegradation comes from observations that exclude all other possible explanations for the fate of an organic contaminant. Circumstantial evidence has provided the main support for the occurrence of in-situ biodegradation in several of the papers previously discussed (Ehrlich et al. 1982, 1983; Goerlitz et al. 1985; Vogel et al. 1981). These reports are the best available documentation of in-situ activity. All have relied in part on the measurement of metabolites unique to microbial activity in the particular circumstances of the studies.

unique biological responses: Behavior unique to biological processes can be used as circumstantial evidence that in-situ biodegradation has occurred. One example of such behavior is the process of microbial acclimation to a new carbon source. Acclimation often involves a readjustment of the levels and types of enzymes (induction or derepression) or species of bacteria in response to the presence of a new substrate. Initially, the concentration of enzyme-adapted bacteria is extremely low, and this is reflected by their minimal impact on the substrate concentration. A number of cell doublings is required before the acclimated population is large enough to dramatically decrease the substrate concentration. The time required between the introduction of a new substrate and the

onset of rapid biodegradation is termed the acclimation lag. Re-introduction of substrate after depletion of the initial dose results in an immediate and more rapid biodegradation response from the acclimated population. Such a sequence of events cannot be explained by abiotic removal mechanisms.

These biological acclimation responses have been used as strongly suggestive evidence of in-situ biodegradation (Herbes and Schwall 1978; Humenick et al. 1982; Kappeler and Wuhrmann 1978; Kuznetsova 1966; Lee et al. 1984; McKee et al. 1972; Naumova 1960; Spain et al. 1980; Wilson et al. 1985). For example, Lee et al. (1984) incubated aseptically collected subsurface material with various aromatic compounds that are typical of the creosote waste that had contaminated some of the locations from which the subsurface material was collected. Bacteria in creosote-contaminated subsurface material degraded the test compounds much more rapidly than bacteria from less-contaminated or uncontaminated subsurface material. Presumably, the bacteria in the contaminated samples had acclimated to the creosote constituents before the sample was collected and therefore were in much larger numbers than in uncontaminated samples. These larger populations could exert a dramatic effect on the substrate concentration due to their greater proportion of acclimated cells. The uncontaminated samples contained far fewer acclimated bacteria and thus were much slower to degrade the fresh substrate.

A major problem encountered not just by groundwater microbiologists, but also by others dealing with environments where potential substrates are at very low concentrations (oligotrophic) or where the contaminant is "biorefractory," is that alternative forms of catabolism may exist. These forms have been categorized under various terms, such as cometabolism, cooxidation, and secondary-substrate utilization, but their major characteristic is that a normally refractory or low-concentration substrate can be degraded when another, utilizable substrate is present (Horvath 1972; McCarty et al. 1981). These possibilities further exacerbate the difficulties in studying groundwater biodegradation.

Despite these complicating factors, an increase in the number of bacteria acclimated to specific contaminants has indeed been correlated with the presence of HCEP contaminants at subsurface sites (Ehrlich et al. 1982, 1983; Goerlitz et al. 1985; Humenick et al. 1982; Jamison et al. 1975; Kuznetsova 1966; Lee et al. 1984; McKee et al. 1972; Naumova 1960; Ogawa et al. 1981; Werner 1985; Wilson et al. 1985). These so called "acclimated responses" have also been observed for non-HCEP wastes (Dockins et al. 1980; Harvey et al. 1984; Leenheer, Malcolm, and White 1976; Olson et al. 1981; Roberts, Schreiner, and

Hopkins 1982; Whitelaw and Rees 1980). The major shortcomings of this type of evidence as reported in the literature are uncertainties with the cell-number estimation methods and lack of parallel estimations from less-contaminated sites that would serve as baseline, control references.

cell-number estimation and sampling: Cell-number estimations obviously depend on the sampling and counting methods. Accurate determination of viable cell numbers has been a long-standing problem in the field of microbial ecology. A variety of methods has been used on subsurface samples including (i) direct counts of samples stained with fluorescent dyes and observed by epifluorescent microscopy (Baker and Mills 1982; Herson and Baker 1982; Hobbie, Daley, and Jasper 1977; Paul 1982; Peele and Colwell 1981; Polenko, Mayfield, and Inniss 1979; Zimmermann, Iturriaga, and Becker-Birck 1978), (ii) scanning electron microscopy (SEM) (Bowden 1977), (iii) standard pour-plate counts (Balkwill and Ghiorse 1985; Jamison et al. 1975; Lee et al. 1984; Ogawa et al. 1981; Slavina 1965; Werner 1985; Whitelaw and Rees 1980), (iv) measurements of cell components (e.g., ATP) for estimating biomass (Federle et al. 1986; Karl 1980; Webster et al. 1985; White et al. 1983), and (v) cellular volume estimates by SEM of Nuclepore-filter-collected samples (Krambeck, Krambeck, and Overbeck 1981). All of these methods depend on the ability of the sampling method to obtain a representative subsurface sample that is not contaminated with surface organisms and that does not affect the viability of the collected organisms.

Representativeness of collected samples is a major concern. It is probable that most bacteria exist in an attached state as biofilms in the subsurface (Harvey et al. 1984; Ventullo et al. 1983). Most investigations rarely have the intention or capability of collecting intact biofilms. Even when biofilm organisms are sampled, the efficiency of extraction from the solid surfaces before counting is rarely reported. Bacteria on the surfaces of individual particles have been observed directly, without prior release (Bouwer and McCarty 1982), although reliable extrapolation of the number of cells per particle to a statistically significant value for the number of cells per cubic centimeter of subsurface material has not been documented.

The majority of studies use well-water samples for determining cell numbers. It is unknown whether these provide a representative proportion of attached and unattached bacteria. Well-water samples may not even reflect the correct range of diversity (Balkwill and Ghiorse 1985). It is not clear if the reported diversity of bacteria in these samples results from species indigenous to the subsurface environment or from those of the well

environment or even the surficial environment (Hirsch and Rades-Rohkohl 1983). One of the biggest problems with these samples is the possibility of contamination from the immediate well environment. Controls are never reported for background levels of contaminant bacteria. For example, lacking are the quantity and types of well-contaminant bacteria that (i) live in the immediate well environment, (ii) are removed with each successive well-volume flush prior to sampling, and (iii) exist in the well water that is finally sampled. It is possible that stable biofilms could exist in portions of the well environment and could serve as a reservoir for continuous supply of background-level microbial or chemical contaminants to well water samples because of gradual sloughing of biofilm material. Even if the background level of these organisms were only 1%, this portion could be the most viable of the collected organisms and thus dramatically affect viable-cell counts. Indeed, one study reported finding only 1% to 10% of the total cells as being viable (Webster et al. 1985).

A number of investigations have estimated cell numbers with direct counts of bacteria retained on Nuclepore filters (Balkwill and Ghiorse 1985; Ehrlich et al. 1983; Harvey et al. 1984; Ladd et al. 1982; Olson et al. 1981; Ventullo and Larson 1985; Wilson and Noonan 1984). Total counts were obtained by staining the fixed cells with acridine orange (AO) and then viewing the preparation under oil immersion by epifluorescent microscopy (Hobbie et al. 1977). Incubation of the cells with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl-5-phenyl) tetrazolium chloride (INT) prior to filtration and staining with AO allowed the counting of viable, respiring cells. An active cell can convert INT to INT-formazan, which appears as a dark spot within the cell under epifluorescent microscopy (Baker and Mills 1982; Herson and Baker 1982; Zimmermann et al. 1978). Despite some advantages when compared with standard plate counts (Herson and Baker 1982), the AOINT direct-count method has some limitations when applied to aquifer material. Noncellular material can give false-positive fluorescence and thus interfere with the interpretation of the AO stain. Chemical reduction of INT can also occur by abiotic mechanisms (Baker and Mills 1982) and does not work well below a minimum cell size (Zimmermann et al. 1978). This latter limitation is especially significant since many subsurface bacteria are expected to be very small after reproducing in an oligotrophic environment. It is also possible that the incubation methods with INT are not sufficiently representative of subsurface respiration rates and therefore could over- or under-estimate those cells that are active in situ.

Many studies have used well-water plate counts to estimate the numbers of acclimated organisms in a contaminated aquifer. Plate counts are probably the least reliable method because the laboratory incubation conditions are radically different from the subsurface environment, especially with regard to solid-phase composition and substrate concentrations. It is likely that many of the isolates that grow on agar medium are not representative of those species that are important in the subsurface. There are too many assumptions necessary to correlate plate counts with the actual number of acclimated organisms present in the subsurface environment.

spatial-sampling evidence: Circumstantial evidence often involves the elimination of other possible explanations by integrating a series of observations or analyses. Some studies are more convincing than others because they have sampled a series of wells along the groundwater flow gradient associated with a source of contamination and the associated plume (Ehrlich et al. 1982, 1983; Goerlitz et al. 1985; Harvey et al. 1984; Leenheer et al. 1976; Roberts et al. 1982; Vogel et al. 1981). For example, a direct correlation was found between the level of contamination from land-disposed, secondarily treated sewage and the numbers of free-living (unattached) bacteria and degree of heterotrophic uptake of simple metabolites in an aquifer (Harvey et al. 1984). The number of bacteria and the rate of glucose uptake decreased in proportion to the increasing distance from the contamination source along a gradient line containing five sampling wells. The profile of data is much more convincing than data from one contaminated site and one uncontaminated site.

aquifer restoration: Another form of circumstantial evidence for in-situ biodegradation is the evocation of a predetermined positive response from the in-situ addition of nutrients or acclimated inocula. Enhancement or stimulation of in-situ microbial activity for the purpose of eliminating groundwater contaminants is a promising method of treatment that has yet to be convincingly documented (see Flathman and Githens 1985; Lee and Ward 1985; Quince and Gardner 1982). Most reports of in-situ enhancement have appeared in the gray literature (e.g., Brown and Norris 1984; Flathman and Githens 1985; Yaniga and Mulry 1984). Even when open-literature reports are considered (Heyse, James, and Wetzel 1986; Jamison et al. 1975; McKee et al. 1972; Werner 1985), none contains sufficient documentation of methods or proof that the enhanced removal was due to stimulated microbial activity. In some cases, the proprietary nature of an investigation for a private company was possibly responsible for the lack of detailed information provided in the final report (e.g., Raymond, Hudson, and Jamison 1976).

In most instances, nutrients supplying nitrogen, phosphorus, or molecular oxygen are added in situ, and an increased disappearance of contaminants is attributed to the effects of stimulating microbial activity. For most of these studies, proper controls were not run to determine if the nutrient addition may have alternatively initiated chemical reactions (e.g., removal by precipitation as phosphate salts) or physicochemical removals (e.g., volatilization). The most convincing report on successful in-situ enhancement demonstrated the disappearance of added nitrate (an electron acceptor) with the concomitant production of molecular nitrogen (i.e., denitrification) and a dramatic increase in cell number presumably supported by oxidation of hydrocarbons present in the oil-contaminated aquifer (Werner 1985).

The purposeful introduction of acclimated microorganisms into a contaminated aquifer has not yet been successfully demonstrated to enhance in-situ biodegradation (e.g., Flathman and Githens 1985). Besides inadequate documentation of methods and poorly designed aquifer-monitoring schemes, there are many problems (physical and ecological) that have probably prevented reliable demonstration of in-situ enhancement of biodegradation from inoculation of a contaminated environment with exotic microorganisms (Brown et al. 1986; Goldstein, Mallory, and Alexander 1985; Lee and Ward 1985). Furthermore, the purposeful introduction of genetically modified microorganisms is strictly regulated and has not yet been allowed in an aquifer.

Probably the most critical problem facing purposeful introduction is the filtering ability of most subsurface solid materials (Brown et al. 1986; Crane and Moore 1984; Gerba 1985; Zoeteman 1985). Wide dispersal of bacterial inocula in the subsurface is dependent on the permeability of the subsurface strata to microbial cells and would be vital to the successful exposure of cells to the dispersed chemical contaminants. Wide dispersal of microbial inocula has not yet been reliably documented in the literature.

In aquifers that are relatively permeable to bacteria, care would need to be taken to sustain the permeability by controlling the size of the inoculum and the onset of rapid growth and biomass accumulation. An excessive concentration of bacteria in the injection water or uncontrolled growth at the site of injection can cause clogging problems that effectively block further dispersal (Brown et al. 1986; McDowell-Boyer, Hunt, and Sitar 1985; Oberdorfer and Peterson 1985). One method of inoculation that offers advantages at maintaining permeability is the introduction of bacterial spores instead of vegetative cells (Jang et al. 1983).

The vast literature on enhanced oil recovery (EOR) contains information directly relevant to microbial aspects of aquifer restoration. In particular, a recent review of the EOR literature (Brown et al. 1986) focused on microbial enhanced oil recovery (MEOR). This area of research has involved numerous studies that investigated both the purposeful introduction of acclimated organisms and the in-situ stimulation of indigenous bacteria in deep well environments. The (MEOR) literature has been largely overlooked by recent investigations of groundwater microbiology and aquifer restoration.

An aquifer restoration project that will potentially have the necessary controls and experimental design to conclusively document in-situ enhancement of biodegradation is under development at the Stanford University Water Quality Laboratory at a test site at the Naval Air Station Moffett Field (Mountain View, CA). Laboratory simulations (Wilson and Wilson 1985) have demonstrated that the contaminant under study, trichloroethylene (TCE), can be degraded to carbon dioxide by methanotrophs that are actively oxidizing methane and propane to carbon dioxide. Five controlled stages to the experimentation may allow definite conclusions. A "conservative" tracer, bromide ion, will be injected into an aquifer uncontaminated by TCE to monitor the natural flowrate and the flowrate artificially created by overt pumping of ground water from an extraction well. Next, bromide and TCE will be injected to monitor the fate of TCE with bromide as an inert reference. This will establish background levels of TCE removal. Then a third injection into the aquifer will contain bromide, TCE, and nutrients to determine whether an increased removal of TCE might occur due to stimulation of microorganisms that may have been previously nutrient starved. A fourth stage of the experiment will involve the pulse injections of methane and oxygen to stimulate an increase in the methanotroph population. The final phase of the experiment will involve the spike (pulse) addition of TCE to the methane and oxygen injection to see if TCE is removed at a significantly greater rate than the background rates of removal determined in the earlier stages. Unfortunately, core samples that could provide credible data on successful increase in the methanotroph biomass are not possible because they would disrupt the artificially induced groundwater flow characteristics. This experiment is an excellent example of the amount of information that could be gathered if regulatory agencies allowed the use of radiolabelled TCE. Indeed, permission to inject unlabelled TCE into an uncontaminated aquifer was contingent upon removal of the "contaminated" ground water at the extraction well followed by air stripping to remove the TCE.

Abiotic controls. A major weakness in most reports on in-situ biodegradation of groundwater contaminants is the lack of controls for abiotic mechanisms that could have accounted for observed disappearance of contaminants or, likewise, appearance of what would otherwise be metabolites or products of metabolism. The actual groundwater environment cannot be controlled, so laboratory simulations that incorporate abiotic removal mechanisms must be used to estimate the portion of in-situ contaminant losses resulting from abiotic processes. The degree of authenticity of these simulations will determine the reliability of assumptions made regarding expected abiotic activities.

dispersion: Many claims of in-situ biodegradation are based on measured disappearances of substrate in situ being greater than those predicted to result from abiotic mechanisms (Bedient et al. 1984; Bouwer et al. 1984; Ehrlich et al. 1983; Godsy, Troutman, and Ehrlich 1983; Goerlitz et al. 1985; Humenick et al. 1982; Reinhard, Goodman, and Barker 1984; Roberts et al. 1982; Schwarzenbach et al. 1983; Wilson et al. 1985). Usually it is assumed that dispersion and sorption will account for most of the abiotic reductions in contaminant concentrations. Estimates of the amount of dispersion or sorption require further assumptions in the design of lab-scale simulations and the interpretation of lab and field data. Because of the numerous assumptions and possibilities for error (sometimes by orders of magnitude), claims of in-situ biodegradation must not be based solely on the reduction of contaminant concentration being greater than that projected by simulation or modeling.

Calculation of in-situ reductions in contaminant concentration because of dispersion is usually based on assumptions concerning the flow rate of the ground water and the in-situ behavior of certain "inert" tracer compounds. Monitoring the flow of contaminant plumes, however, is plagued with difficulties (Josephson 1983), some of which are exacerbated by spatial and temporal variations that exist in an aquifer (Junk, Spalding, and Richard 1980; Spalding and Exner 1980). The most convincing descriptions of subsurface flow are obtained by using numerous sampling wells, although these could in turn alter the flow itself. For example, one study (Patterson et al. 1985) thoroughly studied the flow characteristics along the length of 200 piezometer pipes, but the possibility of flow alteration by the numerous pipes was not discussed. The discontinuities introduced at the pipe and subsurface-strata interface might allow more rapid vertical transport in localized areas. Despite these problems, dispersion is normally quantified by monitoring the reduction in the concentration of a water-soluble compound that is not expected to undergo biological transformation, volatilization, or sorption. In the literature, such an inert tracer

is designated as "conservative." Colligative properties, such as conductivity (Harvey et al. 1984), and inorganic ions, such as iodide (Patterson et al. 1985), chloride (Ehrlich et al. 1983; Reinhard et al. 1984), and sodium (Ehrlich et al. 1982, 1983), have been used as conservative tracers. Comparing the behavior of an inorganic tracer with that of a organic contaminant, however, has many limitations, especially if the organic compound has limited water solubility. In the latter instance, two-phase "flow" might result (Reinhard et al. 1984; Rostad et al. 1985). The possible existence of multiple liquid phases in contaminated subsurfaces also has implications for biological activity, since a predominantly organic phase would represent the opposite extreme of normally oligotrophic aquifers. These extremely heterogeneous microenvironments would present another level of complexity to the groundwater environment.

Probably the best choices as tracers for monitoring dispersion are biorefractory compounds from the same chemical class as the contaminant under study. It can be more easily assumed that these compounds will better simulate the contaminant with respect to sorption and dispersion. Several studies have used biorefractory organic compounds as tracers (Goerlitz et al. 1985; Patterson et al. 1985; Roberts et al. 1982).

Determination of dispersion rates can be directly affected by the abiotic removal mechanism of sorption. It is important to distinguish dispersion removals from abiotic removals by estimating the sorption of the tracer and the target contaminant. The likelihood of sorption and the degree to which a compound will sorb are sometimes estimated by use of the octanol/water partition coefficient (Patterson et al. 1985; Reinhard et al. 1984; Rostad et al. 1985; Schwarzenbach et al. 1983; Schwarzenbach and Westall 1981; Stratton, Namkung, and Rittmann 1983), originally developed for estimating bioaccumulation. Reported values in the literature sometimes have large discrepancies (Goerlitz 1984) probably because of the variety of experimental protocols used to determine the coefficients.

sorption: Sorptive capacities of subsurface solids are often estimated in the laboratory using sorption isotherm experiments (Caron, Suffet, and Belton 1985; Dunlap et al. 1972; Ehrlich et al. 1982; Humenick et al. 1982; Schwarzenbach and Westall 1981; Subba-Rao and Alexander 1982). One weakness of this approach is that the presence of a non-settleable particulate phase is usually not taken into account (McDowell-Boyer et al. 1985). This can yield erroneously low sorption coefficients, but, more importantly, this colloid phase can promote the transport of compounds that would normally be immobilized

by subsurface strata. Care must be taken with estimates of sorptive capacity since the presence of native dissolved organic components (e.g., humic substances) (Caron et al. 1985), colloidal materials (Voice, Rice, and Weber, Jr. 1983), and metal ions in the ground water can affect sorption onto subsurface solids. Metal ions and compounds that are amphiphatic (e.g., fatty acids) or polyfunctional (e.g., humic acids) can increase the solubility of less-polar compounds by ligand formation, emulsification, or extraction, respectively. Ligand formation has been shown to be important for a major class of compounds that play critical roles in HECF wastes (e.g., nitrogen heterocycles) (Stetter, Stamoudis, and Jorgensen 1985). Sometimes, lab isotherm estimates are not considered necessary because the subsurface material was assumed to be saturated by contaminants (Roberts et al. 1982).

volatilization: Abiotic losses from volatilization and chemical degradation are generally considered to be insignificant compared with reduction from dispersion and losses due to sorption and biodegradation. Significant losses from in-situ volatilization is improbable because there is little or no exposure to the atmosphere in subsurface environments (Smith and Dragun 1984); only a static gas phase would exist in the pore-structure of the vadose zone. Even so, volatilization controls are often lacking in lab studies that have controlled for other abiotic mechanisms. Volatilization could be a major transport route if biogas production occurred rapidly or if air were injected into the subsurface as a source of molecular oxygen to promote the growth of aerobes.

Compounds can volatilize from the aqueous phase or from a solid phase. Volatilization of compounds sorbed to solids differs dramatically depending on the moisture and organic carbon content of the surfaces. Only recently has it been recognized that the equilibria established between a gas phase and an aqueous or solid phase can differ (Chiou and Shoup 1985). This is because the presence or absence of water at the surface of carbonaceous or noncarbonaceous surfaces will lead to both normal- and reverse-phase mechanisms of organic-solute partitioning.

chemical transformation: Although little information exists on in-situ chemical reactions (e.g., oxidation/reduction) involving groundwater contaminants, chemical degradation of contaminants is considered to occur too slowly to be of any significance in the interpretation of removal data (Smith and Dragun 1984). Even so, it is still important to control for possible chemical transformations, since they can occur (Parsons and Lage 1985; Schwarzenbach et al. 1985). Chemical hydrolysis under environmental conditions has been reviewed (Mabey and Will 1978).

Sampling controls. In a recent review of the methods used for measurement of in-situ metabolic activity in aquatic environments (Findlay and White 1984), the four main approaches all required sample acquisition. Most evidence supporting claims of in-situ biodegradation rely on samples obtained from the subsurface. In most of these studies, controls are never used to verify that the sample was not contaminated by surface or well-dwelling microorganisms or by compounds from the surface or well. The aseptic collection of intact subsurface samples partly depends on the drilling-method used. Advantages and disadvantages of various well-drilling methods have been discussed (McNabb and Mallard 1984). Common problems that can disturb the integrity of samples during collection or storage have also been discussed (Ghiorse and Balkwill 1983; Wilson et al. 1983).

It is frequently assumed that an aseptically collected water sample from a multiply flushed well is representative of the actual ground water in the vicinity of the well. Evidence is never provided, however, that all of the well-dwelling microbes have been flushed from the system prior to sample collection (e.g., Willis et al. 1975). In cases where organisms were isolated from well-water samples, it was apparently assumed that the isolates were originally located at some distance from the well intake prior to pumping. Such an assumption could be faulty if the particular subsurface solid material acts as a filtration barrier to the movement of bacteria-sized particles.

The choice of sample tubing through which the ground water is pumped or withdrawn can be critical because many tubing materials allow permeation of volatile solutes or sorption to occur (Barcelona, Helfrich, and Garske 1985). It is of critical importance to control for these losses and to reduce their magnitude by using appropriate tubing (e.g., Teflon, stainless steel), while recognizing that even fluoropolymers have the ability to sorb certain organic compounds (Daughton, Jones, and Sakaji 1985). Lack of controls or poor documentation for sampling methods greatly limit possible conclusions (e.g., Norenkova 1966; Pomerants 1966; Slavnina 1965; Smirnova 1957; Zinger 1966).

Laboratory-scale simulations. To avoid problems associated with field studies and in-situ experimentation and measurements, groundwater microbiology and the environmental fate of groundwater contaminants have been primarily investigated in the laboratory. There are a number of factors that have encouraged the prevalence of lab-scale simulations of the groundwater environment, especially since they are easier and less expensive to implement. A major advantage is the ability to obtain mass balances. Lab-scale simulations are more

flexible and allow a variety of controllable operating conditions for investigations of the effects of perturbations to a groundwater system. Because of the ability to control conditions in replicate experimental designs, individual processes operating in the groundwater environment can be isolated in lab-scale simulations and distinguished from other simultaneously occurring processes that affect contaminant fate. Kinetic models of fate can be much more easily and rapidly generated.

A major disadvantage of lab-scale simulations is that their operating conditions are probably very different from the native conditions of the groundwater environment. Indeed, the opposite extreme of field work is the oversimplification epitomized by pure-culture, single-substrate systems. Although pure-culture studies have been extremely useful in the development of microbial ecology and microbiology in general, lab-scale simulations in groundwater microbiology have increasingly emphasized mixed-culture studies involving actual aquifer material and more realistic simulations of environmental conditions (Ehrlich et al. 1982, 1983; Herbes and Schwall 1978; Hutchins et al. 1984b; Kuhn et al. 1985; Lee et al. 1984; McKee et al. 1972; Parsons, Wood, and DeMarco 1984; Pritchard and Bourquin 1984; Rees and King 1981; Spain et al. 1980; Ward 1985; Wilson and Noonan 1984; Wilson and Wilson 1985).

There are several major problems with most mixed-culture simulations. Because most lab-scale simulations show little authenticity when compared with the actual groundwater environment, it is not known whether the data and models generated in the lab are reliable estimates of in-situ behavior. An important unresolved question in lab-scale investigations of groundwater contamination is the minimum level of authenticity required for predictions of actual environment fate. Many lab-scale simulations are termed "microcosms" and fit the loose definition of "any part of an ecosystem that may be subject to laboratory control due to its reduction in size or complexity" (Bengtsson 1985). Most of these simulations, however, would not fit the more strict definition (Pritchard and Bourquin 1984) that requires a level of authenticity based on the collection and incubation of an intact piece of the subsurface that is self-sustaining under nearly identical environmental conditions (e.g., seasonal fluctuations in moisture, aeration, temperature, and solute characteristics and concentrations).

Since many different experimental designs have been used to simulate the groundwater environment, it is very difficult to compare results of one design with those

of another, even when both are intended to simulate the same environmental location (Van Veld and Spain 1983). The problems of low authenticity and lack of comparability add to the difficulties in estimating the margin of error in fate predictions made from data obtained in lab-scale simulations. It is very important to attempt to verify lab observations with parallel field studies. One of the few such studies was the successful verification under field conditions of the accelerated reduction in concentration of pesticide-contaminated soil by inoculation with specifically adapted bacteria (Barles, Daughton, and Hsieh 1979). Such coordinated field and lab investigations could help estimate the range in error associated with reliance on lab-scale simulation data.

Integrated lab and field studies on the fate of groundwater contaminants have provided mixed results regarding the agreement between lab and field behavior of the contaminants. Although the behavior of dimethyl- and dichlorobenzenes in laboratory simulations of river water/groundwater infiltration was qualitatively the same as observed at a field site (Kuhn et al. 1985), there were difficulties in transferring lab-determined rate constants to the field. A lab-scale, model aquifer system (Horvath and Elcan 1978) successfully predicted the apparent field fate (Leenheer et al. 1976) of liquid wastes that were injected into deep wells. On the other hand, serum-bottle studies on the methanogenic decomposition of phenolic compounds derived from creosote wastes (Godsy, Goerlitz, and Ehrlich 1983) gave lower removals for some phenolic compounds than the removals observed under field conditions (Ehrlich et al. 1982, 1983). It was possible that the lack of sufficient solid surfaces in the serum bottles could not support the type of biofilm expected to exist within the extensive surface area provided by the subsurface solids in the field. Lab studies that demonstrated mixtures of alkyipyridines to be biodegradable (Rogers et al. 1985) did not accurately predict the field fate of these compounds; complex mixtures of alkyipyridines were found to persist in groundwater environments contaminated by oil shale wastewaters (Riley et al. 1981).

An important feature of the differences between lab simulation results and field behavior might be the amount of sample disruption. The importance of using intact sediments versus sediment slurries (e.g., Wilson et al. 1983) has been discussed in a review of methods used for determining in-situ metabolic activity (Findlay and White 1984). Many of the inconsistencies between field and lab observations could be directly related to the integrity of the indigenous biofilm. Sediment slurries can be expected to extensively disrupt biofilms and possibly their ability to effectively simulate the biodegradation of contaminants at low concentrations in oligotrophic environments. As stated in this review

(Findlay and White 1984), it is evident that the complex interactions of different processes and environmental conditions in heterogeneous subsurface environments require methods and techniques that do not disturb the metabolic processes that they attempt to measure.

III. CONCLUSIONS

Current knowledge of the enormous habitat extremes and diverse metabolic capabilities of microorganisms could easily lead to a justifiable extrapolation that they play a crucial role in the environmental fate of organic compounds in the subsurface environment. Although this is quite probable, there is a definite lack of conclusive evidence for (i) the ubiquitous occurrence of microorganisms in the subsurface environment, (ii) in-situ activity of indigenous microorganisms (should they exist), (iii) effective dispersal and functional activity of exotic microorganisms, and (iv) in-situ biodegradation of xenobiotic compounds.

It appears certain that bacteria do exist in the subsurface, but only a few reports provide well-documented descriptions of their in-situ subsurface activity. Their origins (indigenous vs. exotic) and levels of activity are still unclear. The in-situ occurrence of biodegradation has been conclusively detected in only a few instances. Of these studies, few are directly relevant to the problem of groundwater contamination by organic compounds from hydrocarbon extraction/conversion processes. The enhancement of indigenous microbial activity or the inoculation of aquifers with pre-adapted or genetically modified bacteria appear to be promising approaches to the economical restoration of contaminated aquifers in the least environmentally disruptive manner, but this is purely at a speculative stage.

Most reports that claim or refer to in-situ biotransformations of groundwater contaminants lack one or more of the following: sufficient documentation, reliable sampling methods, suitable controls for ruling out competing, abiotic contaminant-removal processes, or sufficient authenticity in laboratory-scale simulations. Future research efforts will require dramatic increases in funding to support more extensive and reliable sampling programs, increased authenticity in laboratory simulation experiments, and a greater integration of laboratory and field studies. Only after the completion of substantially more research, can we expect to make reliable predictions of the fates of groundwater contaminants and design appropriate aquifer-restoration schemes.

IV. RECOMMENDATIONS FOR FUTURE RESEARCH

(1) Large-scale funding is necessary to provide conclusive results required for an adequate understanding of the in-situ fate of organic contaminants in groundwater

environments. Minimal funding of projects may be worse than no funding because inconclusive results only serve to obscure the literature data base that is relied upon for fate predictions and future experimental designs. The best available technology for groundwater sampling should be considered an absolute requirement for assessing the in-situ activity of indigenous, nutrient-induced, or purposefully introduced microorganisms. This technology is expensive to implement and project funding should not limit the number of unique samples required for conclusive demonstrations of in-situ activity.

(2) Supplementary funding of existing in-situ bioreclamation projects should be encouraged for the purpose of obtaining better documentation. Most bioreclamation reports end up in the "gray literature" because it was not necessary for the contractor to conclusively prove that microorganisms removed the targeted groundwater contaminants. In some cases, conclusive proof might only have required a few additional samples with proper controls and more carefully documented methodology. In the interest of establishing a more complete scientific data base from the refereed literature, a cooperative effort between private consulting firms, academe, and respective private or government funding agencies would benefit all concerned.

(3) New bioreclamation projects should be funded when the contractor has clear ideas as to how enhancement of in-situ activity could be achieved or when the proper agencies grant permission for purposeful-introduction experiments (i.e., inoculation or seeding with acclimated or genetically modified microorganisms).

(4) Research into more reliable groundwater sampling methods should be pursued with the additional intention that standardized sampling methods might evolve. A side benefit could be the reduced cost of sampling because of mass production of standardized equipment. For example, aseptically obtained core samples (e.g., see Wilson et al. 1983) should be required when indigenous microorganisms are being characterized because it is presently the only reliable sampling method.

(5) New methods of chemical analysis using remote sensing should be evaluated and implemented for groundwater contaminant identification and quantification. For example, the feasibility of using fiber optics for monitoring groundwater contaminants in situ has been demonstrated (Hirschfeld et al. 1984).

(6) In-situ mass balances using carbon-14 labelled substrates could greatly expand our knowledge of the fate of organic contaminants and further allow us to distinguish between various removal mechanisms. Since the introduction of radiolabelled compounds into the groundwater environment is prohibited, it will be necessary to alter current regulatory policies to allow for occasional, carefully controlled introductions for selected studies. Special methodology to demonstrate the safety and feasibility of such mass-balance techniques in situ will probably be required before permission can be granted.

(7) Because of the very large expense required for reliable field studies, it is of extreme importance that we improve our ability to accurately model and predict the behavior of groundwater contaminants based upon lab-scale simulations. To accomplish this, lab studies must be integrated with field studies whenever possible. Basic data will need to be obtained from simplified, lab-scale systems using a limited number of appropriate model compounds (e.g., see Daughton 1986; Zachara et al. 1984) to simulate microbial degradation of complex mixtures of trace organic compounds in ground water.

(8) Baseline data are extremely important for uncontaminated groundwater environments likely to be contaminated by future development of hydrocarbon extraction/conversion process (HECP) facilities. This is important to establish native levels of compounds that would perhaps be introduced later by a contaminant plume. It will also be advisable to characterize any indigenous microbial activity or potential for activity before perturbation of the environment by HECP facilities. Such data might also help in HECP site location, process designs, and establishment of waste-treatment criteria.

(9) Fundamental research should be encouraged in various areas of biodegradation where a paucity of information or gaps in the literature exist. Such areas directly relevant to groundwater microbiology include: (i) the metabolism of complex mixtures of organic substrates by mixed populations of bacteria, (ii) biodegradation at low substrate and biomass concentrations, (iii) interactions of contaminants and bacteria in biofilms and on surfaces, and (iv) long-term biotransformations that might occur in very slow-moving groundwater systems.

(10) Distantly related bodies of literature (e.g., enhanced oil recovery literature; biofouling literature) should be searched and reviewed for applicability to solving problems that have been described in the current groundwater literature. For example, a vast literature on enhanced oil recovery contains information on solving similar problems in

subsurface environments (e.g., introduction of surface-produced bacterial inocula into the subsurface (Brown et al. 1986; Jang et al. 1983)).

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Table I. Most Abundant or Frequently Occurring Compounds from the Major Chemical Classes Occurring in Oil Shale Process Streams ^a

| <u>Chemical Class</u> | <u>Major Compounds</u> |
|---|--|
| <u>Hydrocarbons</u> | |
| alkanes | methane; <i>n</i> -decane |
| alkenes | <i>n</i> -1-decene; 1-pristene |
| alkylbenzenes | benzene; <i>o</i> -xylene; toluene; ethylbenzene |
| diaromatics/PAHs | naphthalene; acenaphthene |
| <u>Hydroxyls</u> | |
| alkanols | 2-propanol |
| hydroxybenzenes | phenol; 2-hydroxytoluene (<i>o</i> -cresol) |
| <u>Carbonyls</u> | |
| alkanals | formaldehyde; acetaldehyde |
| alkanones | acetone; 2-butanone |
| cycloalkanones | cyclohexanone |
| alkanoic acids | heptanoic acid |
| alkanedioc acids | pentanedioc acid |
| aromatic acids | benzoic acid; 2-methylbenzoic acid |
| amides (acylamines) | acetamide |
| amides (lactams) | 1-methyl-2-pyrrolidone; 2-piperidone |
| urea | urea |
| <u>Amines (primary)</u> | |
| alkylamines | methylamine |
| arylamines | aniline; 2-ethylaniline |
| <u>Heterocycles (pi-excessive)</u> | |
| pyrroles | pyrrole; 2,5-dimethylpyrrole |
| benzopyrroles | indole; <i>N</i> -methylcarbazole |
| thiophenes | thiophene; 2,5-dimethylthiophene |
| furans | benzofuran |
| <u>Heterocycles (pi-deficient)</u> | |
| pyridines | 2-methylpyridine; 2,4,6-trimethylpyridine |
| oxo-pyridines | 2-pyridone |
| naphthyridines | quinoline; 2-methylquinoline |
| fused-ring | benzoquinoline; acridine |
| muti- and mixed- | 5,5'-dimethylhydantoin ^b |
| <u>Cyanides</u> | |
| hydrogen cyanide | hydrogen cyanide |
| alkylcyanides | acetonitrile |
| arylcyanides | benzonitrile |
| <u>Cyanates</u> | |
| thiocyanate | thiocyanate ion |

Table. I (continued)

Organosulfur (nonthiophenic)

| | |
|----------------------|------------------|
| oxo | carbonyl sulfide |
| thiols | methanethiol |
| disulfides | carbon disulfide |
| sulfides (thiolanes) | dimethylsulfide |

Organometal(loid)s

| | |
|----------------|-----------------|
| alkylmercury | dimethylmercury |
| alkylarsonates | methylarsonate |

^a From Daughton (1986); ^b a possible major constituent.

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