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Review

Subsurface microbial ecology and bioremediation*

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Abstract

A minireview of subsurface microbial ecology as it relates to ground water contamination and remediation is presented. Microorganisms have been detected unequivocally at depths of 500 to 600 m below the surface. Microbial numbers and activity are higher in sandy transmissive sediments than in those with high clay content and low transmissivity. Many of these organisms are active and can metabolize a variety of organic compounds which are of environmental concern. Biodegradation in the subsurface is favored by the presence of acclimated organisms and essential nutrients and the absence of toxicants and inhibitors. Natural bioremediation in the subsurface is enhanced by transporting an electron acceptor and essential nutrients to microorganisms in the zone of contamination. A better understanding of microbial processes in the subsurface may provide better solutions to ground water contamination problems.

Introduction

The biosphere under the root zone is largely uncharacterized in terms of ecological diversity and importance in geochemical processes and more recently, in the fate of environmental pollutants. Lack of interest in subsurface microbial ecology is probably a result of early investigations which indicated that bacterial numbers decreased with depth below the surface [1]. In addition, the expense and logistical difficulties of collecting representative samples have steered research away from subsurface microbiology [2]. As a consequence, it was assumed that the subsurface below the root zone was generally void of life until scientists at the United States Environmental Protection Agency readdressed the question [2,3]. The results of renewed investigations

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of the subsurface indicate that subsurface microorganisms exist and can be metabolically active. The heretofore relatively unexplored subsurface may provide a better understanding of biogeochemical processes, harbor organisms with novel metabolic pathways and provide solutions to ground water and subsurface contamination. The following sections will concentrate on subsurface microbial ecology as it relates to subsurface remediation.

Numbers, distribution and activity of microorganisms

Below the rhizosphere where the terrestrial environment is no longer classified as soil, microorganisms have been found unequivocally at depths of 500 to 600 m [4]. Although earlier works suggested that microorganisms could be found at even greater depths, the results of these studies were considered equivocal because methods for sample collection did not exclude the likelihood of contamination from the surface and drilling operations [2,5]. After development of methods that excluded contamination of shallow subsurface samples around 1980 [6,7], credible data on the numbers, diversity and distribution of microorganisms in the shallow subsurface were reported. In addition, analyses of samples of subsurface material instead of ground water was emphasized because water drawn from wells may contain nonindigenous microorganisms [8]. Initial investigations were conducted to determine the microbial ecology of shallow uncontaminated and contaminated subsurface material, the zone from the rhizosphere to about 10 to 20 m from the surface. Later investigations included samples from the uncontaminated deep terrestrial subsurface at depths of 500 to 600 m, which required specialized sampling equipment [9,10].

In general, the numbers, types, and distribution of microorganisms in shallow and deep subsurface sediments are similar, depend on subsurface characteristics, and are microsite specific. Investigations of the microbial ecology of the shallow subsurface have indicated that bacteria are the predominant type of microrganism present [7,11–14], although protozoa, algae and fungi have been detected as well [15,14,16]. Studies conducted using samples from the deep subsurface also have indicated the presence of the same inhabitants, with bacteria predominating [17–19]. In shallow and deep samples of subsurface materials, direct counts of bacteria are fairly uniform with depth and range from about 10^6 to 10^7 cells/g dry weight [7,11,12,17,20]. Direct counts in this range are lower than those of bacteria in samples of surface soil, around 10^8 to 10^{10} cells/g dry soil [21], and may be explained by the oligotrophic nature of most uncontaminated subsurface environments [5]. Viable counts are usually less than direct counts and range from nondetectable to as high as the direct count [7,11,12,15,17,20,22]. However, within the different strata of a subsurface profile, the bacterial populations may vary, with higher numbers and activity detected in sandy aquifer sediments rather than those high in clay [15,17,19,20,23,24].

TABLE 1

Microbial ecology of ucontaminated and contaminated samples from the same sites

Spill	Variable	Uncontaminated	Contaminated	Reference
Creosote[a]	Predominant cell type	Gm^+	Gm^-	[13]
	Biomass[b] (nmol/GDW[c])	0.6(0.5)	1.0(0.8)-1.9(0.5)[d]	[13]
	Direct Counts (10^6/GDW)	1.2(1.2)-16(8)	8(2)	[25]
		1.4(0.6)-2.3(0.9)	1.3(0.5)-2.4(0.8)	[26]
			0.9(0.4)-2.1(0.8)	
	Viable counts[e] ($\times 10^6$/GDW)	$<10^2$-0.1(0.02)	$<10^2$-2.9(0.2)	[26]
	ATP (ng/GDW)	0.017	0.009	[25]
Coal tar	Direct counts ($\times 10^6$/GDW)	1-100	1-700	[14]
	Viable counts ($\times 10^2$/GDW)	1-100	1-10,000	[14]
	Protozoa (No./GDW)	<50	<50-19,000	[14]
	Fungi (No./GDW)	10	10	[14]
	Actinomycetes (No./GDW)	10-1000	10-10,000	[14]
Unleaded	Viable counts[e] ($\times 10^6$/GDW)	1.6(0.1)-1.8(0.5)	0.3(0.03)-65.5(9.2)	[27]
Gasoline jet fuel	Direct counts ($\times 10^6$/ml)	0.001-0.02(0.008)	0.7(0.1)-13	[28]
	Viable counts[e] ($\times 10^3$/ml)	<1	62-1,400	[28]
Oil products	Glucose uptake V_{max} (μg/L h)	0	0.2	[29]

[a]All measurements of microbial numbers and activity in samples from a creosote spill are from the same site.

[b]Biomass measured as total extractable phopholipid.

[c]Range of measurements can represent different depths in unsaturated and saturated zones and for the contaminated samples, different concentrations of contaminants.

[d]GDW: grams of dry weight of subsurface material.

[e]Range of counts on different types of media.

The microbial ecology of aquifers contaminated with organic pollutants is strikingly different from that found in uncontaminated aquifers. Provided that the nature or concentrations of pollutants are not toxic and there are sufficient nutrients to support growth, the addition of organic contaminants to the uncontaminated subsurface may stimulate microbial growth and activity. A comparison of microbial numbers and potential activity in samples of uncontaminated and contaminated materials from the same site has been conducted by several investigators (Table 1). In general, environmental spills of organic compounds may increase microbial numbers and activity.

Biodegradation in the subsurface

The microflora from uncontaminated and contaminated shallow and deep subsurface materials has been reported to metabolize a variety of naturally-occurring organic compounds including carbohydrates, amino acids, organic acids, methane, cellulose, and lignin-type compounds [30–32]. In addition, compounds from several classes of industrial chemicals that have been shown to biodegrade in shallow subsurface material include petroleum-derived hydrocarbons [17,26,27,30,33–35], chlorinated aliphatic solvents [31,36], phenols [37–39], and polar solvents [39–42]. Studies of the biodegradation of industrial chemicals in the deep subsurface have been limited; however, phenol [43], naphthalene, toluene, the xylene isomers, dibenzothiophene, *p*-cresol [44], nitrogen-containing aromatic compounds [45], and trichloroethylene [19], have been biodegraded by microorganisms from the deep subsurface.

Factors which may affect biodegradation in the subsurface

The same variables which affect biodegradation in terrestrial and aquatic environments also apply to the subsurface. Conditions which favor biodegradation include the presence of acclimated microorganisms, adequate substrate concentration and availablity, the presence of essential nutrients, the absence of toxicants and inhibitors, and appropriate values for pH, temperature, salinity and osmotic pressure [46].

Acclimation is defined as the amount of time between exposure of microorganisms to a substrate and detection of substrate biodegradation. Acclimation may occur as a result of an increase in the number of contaminant-degrading organisms, genetic changes which confer degradation capabilties, enzyme induction, and depletion of a substrate which is preferentially metabolized [47]. Detection of pollutant biodegradation within a relatively short incubation period (days to weeks) also has been reported for samples of uncontaminated subsurface material [7,38,48], suggesting that a lengthy prior exposure to the contaminants before biodegradation can ensue is not required. However, microorganisms in uncontaminated environments may incorporate more of a

contaminant into biomass rather than mineralize it [30]. As a result of lengthy exposure to contamination, it has been reported that subsurface microorganisms may shift their metabolism from less cellular incorporation to more mineralization of the contaminants [48]. Investigations which compare contaminant biodegradation in samples of uncontaminated and contaminated material from the same site can be used to indicate the presence of acclimated microorganisms and the potential for biodegradation of subsurface pollutants (Table 2).

The absence of biodegradation in the subsurface may be a result of the presence of toxins or inhibitors. The lack of biological activity in samples collected from deep subsurface material contaminated with trichloroethylene was thought to result from the high concentrations of contamination (>200 mg/kg sediment and >300 mg/L pore water) present [19]. In another study, mi-

TABLE 2

Biodegradation of organic compounds in samples of uncontaminated and contaminated subsurface material from the same site

Compound	Biodegradation[a]		Reference
	Uncontaminated	Contaminated	
Naphthalene	no	yes	[33]
	no	yes, no[b]	[26]
	no	yes, no	[14]
2-Methylnaphthalene	no	yes	[33]
Dibenzofuran	no	yes	[33]
Fluorene	no	yes	[33]
Acenaphthene	no	yes	[33]
1-Methylnaphthalene	no	yes	[33]
Phenanthrene	no	yes, no	[26]
	no	yes, no	[14]
Benzene	no	yes	[49]
	no	yes	[27]
Toluene	no	yes	[49]
	yes	yes	[27]
	yes	yes[c]	[48]
Ethylbenzene	yes	yes	[27]
m-Xylene	no	yes	[49]
	yes	yes	[27]
o-Xylene	no	yes	[49]
	no	no	[27]

[a] Disappearance of parent compound or mineralization.
[b] Samples from several zones and locations in the subsurface were analyzed.
[c] More was biodegraded in the contaminated than uncontaminated samples.

neralization of naphthalene, phenanthrene, and glucose was detected in samples from the saturated but not the unsaturated zone of subsurface material heavily contaminated with creosote; the lack of activity was thought to result from toxic concentrations of creosote present in the unsaturated zone [26].

Nutrient availabilty also may limit biodegradation in the subsurface. Unlike surface environments, the subsurface microflora rely on the transport of nutrients and electron acceptors from ground water recharge from rivers and streams or percolation from the surface, which are most often slow processes. As a result, biodegradation in the subsurface may be limited by the transport of essential nutrients and electron acceptors to the microorganisms [33], which is ultimately dependant on the permeability of the formation. Several investigators have reported that the addition of inorganic nutrients to samples of subsurface material enhanced or had no effect on contaminant biodegradation [26,27,38]. Most likely, the effect of nutrient amendments depends on the native fertility of the subsurface material.

Biodegradation potential in saturated sediments appears to be related to subsurface characteristics rather than to depth or dissolved organic carbon content [43]. As with numbers, types, and distribution of microorganisms, microbial activity and biodegradation potential are higher in sandy transmissive sediments than in those with high clay content [19,43]. Investigations of microbial activity in the subsurface have indicated that biodegradation potential of acetate and phenol is positively correlated to viable cell counts and pH and negatively related to clay content [43]. However, these correlations were not observed for more recalcitrant compounds such as aniline, quinoline, and pyridine, for which other factors may be involved in their biodegradation [45]. Similarly, biodegradation potential of methanol and phenol in samples of surface soil and subsurface material was positively correlated with viable cell counts, whereas a negative correlation was observed for the more recalcitrant compound, *t*-butyl alcohol [39].

Although many organic pollutants have been shown to biodegrade under aerobic as well as anaerobic environments, biodegradation, when it occurs, is usually faster when oxygen (O_2) is used as the terminal electron acceptor. In addition, initiation of the major biodegradative pathways for many organic pollutants, especially aromatic hydrocarbons, requires O_2. Even readily metabolizable compounds such as carbohydrates have resisted biodegradation in environments devoid of O_2 [50]. The results of several laboratory and field experiments have shown that the presence or addition of O_2 enhances biodegradation of many of the pollutants found in the subsurface. Biodegradation of several polycyclic aromatic hydrocarbons was enhanced by the addition of O_2 to the subsurface and samples of ground water collected from a site contaminated with creosote [51,52]. At this same site, transport of O_2 to contaminant-degrading microroganisms in aquifer material contaminated with creosote was thought to control the size and shape of the resulting plume [33,53]. In a field

experiment conducted in a shallow, unconfined sand aquifer, biodegradation of benzene, toluene, and the xylene isomers was controlled by the availabilty of dissolved oxygen, and the compounds persisted in layers which were oxygen-poor [54].

Contrary to past research that indicated that O_2 is required in the biodegradation of aromatic hydrocarbons [55], more recent studies have shown that these compounds can be metabolized under anaerobic conditions. Several monoaromatic compounds have been reported to biodegrade under methanogenic [56,57], denitrifying [58,59], sulfate-reducing [60], and ferric iron-reducing conditions [61]. A better understanding of the fate of these compounds under anaerobic conditions is warranted because unsaturated and saturated subsurface materials frequently are driven anaerobic as a result of a contamination event. In addition, use of electron acceptors other than O_2 may be advantageous because of the limited solubility of O_2 in water.

Application of microbial ecology to subsurface bioremediation

Many contamination events may go unnoticed because of natural bioremediation [62]. When the rate of natural bioremediation is not fast enough to prevent the spread of contamination, resulting in health and environmental risks, intervention by one of many remedial techniques will be required. Bioremediation is one of those techniques and usually is used in conjunction with several other remedial options. Subsurface microorganisms were stimulated to bioremediate ground water contaminated with hydrocarbons by R.L. Raymond and coworkers as early as 1974 [63]; however, it was not until the early 1980s that the U.S. Environmental Proctection Agency launched a major program to investigate the potential role of microorganisms in the fate of subsurface contaminants [64]. Until then, the potential of subsurface bioremediation as a remedial option was not considered by federal agencies or industry. Most of the bioremediation technologies that since have been developed are variations of those used by Raymond and his coworkers. In addition, most of these refined processes have been used mainly to bioremediate hydrocarbons.

Application of bioremediation to the subsurface involves designing a system that will provide limiting nutrients and an electron acceptor to the microorganisms in the zone of contamination [65]. The system can be designed to treat the unsaturated or saturated zones or both zones simultaneously. Treatment of the unsaturated zone may be accomplished using several methods: by 1) percolating a nutrient solution from the surface down into the unsaturated zone using an infiltration gallery, 2) raising the water table so that a nutrient solution can be perfused through the affected area, and 3) bioventing, a process which combines soil venting with biodegradation and is used for compounds which can be volatilized relatively easily [66]. Figure 1 was designed as a composite to illustrate the three methods that can be used for remediating the

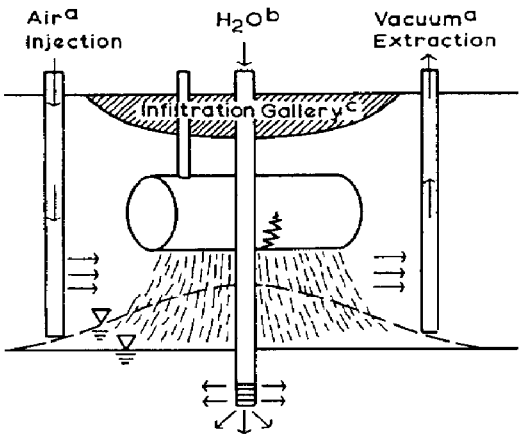

Fig. 1. Composite diagram of different treatment schemes for contamination of the unsaturated zone: (a) vacuum extraction for bioventing, (b) water injection to raise water table, and (c) infiltration gallery to supply nutrients and electron acceptor.

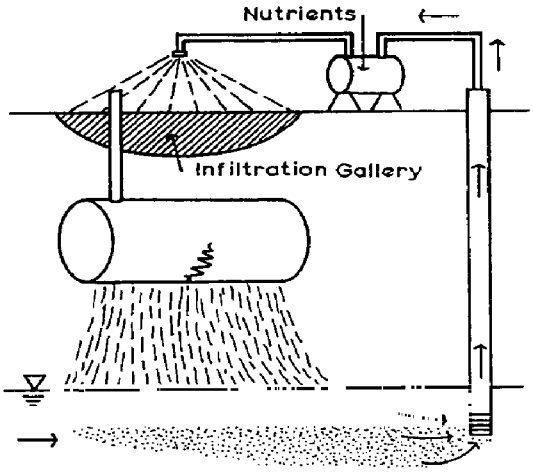

Fig. 2. Schematic diagram of unsaturated and saturated zone treatment in a closed-loop system.

unsaturated zone; this figure should not be used as a basis for process design. The saturated zone may be treated by perfusing the contaminated area with a nutrient solution using injection wells or injection and recovery wells in a closed loop system. Treatment of both the unsaturated and saturated zones simultaneously (Fig. 2) can involve the use of infiltration galleries and recovery wells [65].

All of the above methods, except for bioventing, involve dissolving the electron acceptor, usually oxygen, in water before addition to the subsurface. Using the treatment of monoaromatic hydrocarbons in the bioremediation scenario, large amounts of water will be required to degrade the contaminants because of: (1) the low solubilty of oxygen in water, about 8 mg/L when air is used and

about 40 mg/L when pure oxygen is used (depending on temperature), and (2) the 2:1 ratio of oxygen to hydrocarbon that will be required [62]. As a result, hydrogen peroxide, which is infinitely soluble in water, has been used as a source of oxygen in subsurface bioremediation [67]. Hydrogen peroxide decomposes to yield $\frac{1}{2}O_2$ and H_2O so that concentrations as low as 100 mg/L can provide more O_2 per unit volume than using either air or pure O_2. However, peroxide concentrations as low as 100 mg/L can be toxic to microorganisms [68]. To avoid toxicity, peroxide is added in a step-wise manner from about 50 to as high as 1000 mg/L, to allow the subsurface microflora to adapt to the oxidant. Other problems associated with the use of peroxide include rapid decomposition and off gassing of O_2 to the surface and/or plugging of the region undergoing treatment [69]. Bioventing is another approach to increasing the supply of O_2 to the subsurface because more O_2 can be transported in air than water [66].

Also promising is the use of nitrate as an electron acceptor during bioremediation of the subsurface. The use of nitrate alone or in combination with oxygen is attractive because nitrate is more soluble than O_2 in water. Although denitrification was once thought to occur under strict anaerobic conditions, combinations of O_2 and nitrate to enhance biodegradation may be possible [59,70,71]. In the presence of both, O_2 could be used to initiate biodegradation while nitrate could serve as the terminal electron acceptor. Subsurface bioremediation using nitrate as a terminal electron acceptor in the field has been attempted [72–74].

Innovative approaches to subsurface bioremediation

By studying the niche of microorganisms, several innovative approaches to subsurface bioremediation have been developed or proposed. These include the cometabolism of chlorinated aliphatic solvents, transport of microorganisms through the subsurface to aid in contaminant removal, and the production of bioemulsifiers and biosurfactants by subsurface microorganisms.

The process of cometabolism, during which microbial growth results from metabolism of a primary substrate and a secondary substrate is fortuitously metabolized, has been exploited in attempts to remediate materials contaminated with the chlorinated aliphatic solvents. Cometabolism of the chlorinated compounds is effected by broad-specificity mono- and dioxygenases in bacteria which grow on certain hydrocarbons [75–79]. The ammonia monooxygenase of the autotroph *Nitrosomonas europea* is thought to oxidize many chlorinated compounds as well [80]. Trichloroethylene (TCE) also is cometabolized aerobically by heterotrophic enrichment cultures from subsurface material contaminated with the solvent; TCE biodegradation occurs after growth has ceased in cultures amended with methanol, methane, propane, and tryptone-yeast extract as energy sources [81]. Cometabolism of the chlorinated aliphatic sol-

vents in the field has been attempted by stimulating the growth of indigenous methanotrophs with methane and oxygen [31]. The methanotrophs contain a broad-specificity enzyme, methane monooxygenase, which oxidizes the chlorinated compounds [75,76].

The addition of microorganisms to the subsurface during bioremedial processes would be advantageous in situations where contaminant-degrading organisms are absent. Although microorganisms have been added during subsurface bioremediation operations, their contribution to contaminant removal has not been differentiated from that of the indigenous microflora [82]. For added microorganisms to be effective in degradation, they must be transported through the subsurface to the zone of contamination, colonize and grow in the subsurface matrix, compete with the indigenous microflora for nutrients, and maintain their ability to degrade the contaminants. Properties of the subsurface matrix and the organism will affect its transport. Matrix properties which favor transport include large grain size and related high values of hydraulic conductivity and the presence of cracks or fissures which allow channeling [83–87]. Organismal properties which may affect transport include the size, shape, motility, condition, and stickiness of the cells [84,87–89]. In addition, microbial transport may be enhanced when the cells are injected in a low ionic strength solution to reduce adsorption [87,90]. It has been demonstrated in the field that microorganisms can be transported through aquifers [91,92], however, not for the purpose of contaminant degradation.

Another innovative approach to subsurface bioremediation is the exploitation of bioemulsifier and biosurfactant production to enhance removal of contaminants sorbed and/or entrained in the subsurface matrix. Although the dissolved phase of the contaminants is the most easily treated, the sorbed and entrained phases often represent the majority of the contamination and are extremely difficult to remediate. Biosurfactants and emulsifiers could be exogenously supplied or produced in situ or organisms producing these compounds could be transported to the zone of contamination to enhance contaminant extractability and bioavailablity. The presence of these types of organisms in the subsurface has been reported. A survey of samples of biostimulated (nutrient and oxygen addition), contaminated and uncontaminated subsurface material from a site contaminated with aviation fuel indicated that bioemulsifiers were present in all samples; however, the biostimulated zone contained the best emulsifiers, the contaminated zone contained the greatest diversity of emulsifiers, while the uncontaminated zone contained the poorest emulsifiers [93].

Summary

Research investigating the microbial ecology of the subsurface has indicated that microorganisms exist unequivocally at depths of 500 to 600 m below the

surface. Although bacteria are the predominant forms of microorganisms present, protozoa, algae, and fungi also have been detected. The subsurface microflora is able to metabolize a variety of naturally-occurring and industrial chemicals; however, biodegradation of many organic compounds may be site and even microsite specific. The availability of dissolved oxygen has been found to be the major factor limiting biodegradation in the subsurface. Subsurface bioremediation usually involves transporting oxygen and nutrients to the indigenous microflora in the zone of contamination. Most subsurface bioremediation operations have involved the treatment of hydrocarbons. Innovative approaches to subsurface bioremediation include the use of alternate electron acceptors, cometabolism to aerobically degrade the chlorinated aliphatic solvents, transport of contaminant-degrading organisms in the subsurface and stimulation of biosurfactant production by the subsurface microflora. A better understanding of the microbial ecology of the subsurface may provide better answers and solutions to ground water contamination problems.

Disclaimer

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