Ref: In G. Bitton et al., ed., Encyclopedia of Environmental Microbiology, 6: 3194-3202. John Wiley & Sons, Inc., New York, 2002.

# TRACERS IN GROUNDWATER: USE OF MICROORGANISMS AND MICROSPHERES

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Microorganisms have been deliberately injected into groundwater environments for well over a hundred years. Reasons for the additions include increasing oil recovery from less permeable zones, increasing rates of organic contaminant biodegradation, a better understanding of the hydrologic properties of aquifers, particularly those characterized by a high degree of physical heterogeneity, a better understanding of the factors controlling the transport and (or) survival of pathogenic, genetically engineered, or other non-indigenous microorganisms in groundwater systems, and assessments of the vulnerability of an aquifer to microbial (pathogen) contamination. This chapter will focus on addition and recovery studies in which microorganisms are used as tracers in order to gain information about subsurface microbial transport behavior, biorestoration potential, geohydrology, or vulnerability to microbial contamination of groundwater systems. Because of the importance of microbial-sized microspheres to the overall understanding of subsurface microbial attachment and transport behavior and of the hydrologic properties of different types of aquifers, the uses of microspheres as microbial-sized tracers are also discussed.

## HISTORY OF MICROBIAL TRACERS IN GROUNDWATER

The first published studies involving the use of microorganisms as tracers in injection and recovery studies of groundwater systems occurred in the latter part of the 19th century. In the earliest studies, pigment-producing bacteria were used to delineate flow paths in karst and fractured-rock aquifers (1,2). During the following century, other types of microorganisms were employed, including enteric viruses, bacteriophages, yeast, and protozoa. However, the use of human and animal viruses as tracers in groundwater studies has been limited because of safety and water quality considerations. There are a number of reviews focusing on the use of microorganisms as groundwater tracers. The interested reader is referred to Keswick and coworkers (3) and Gerba (4) for brief descriptions of microbial tracer studies conducted before 1981, to Harvey (5) for a history of microbial tracers up to 1997, to Hötzl and coworkers (6) for descriptions of studies in which microbial tracers are used in a number of geohydrologic studies involving granular and karstic aquifers, and to Rossi and coworkers (7) for a discussion of the use of viruses in groundwater studies.

### Bacteriophages

Following their first use as a surface water . tracer as described by Wimpenny and coworkers in 1972 (8), bacteriophages (bacteria-specific viruses), often referred to simply as *phages,* were employed widely as tracers in groundwater applications. A modest number of phage tracer experiments involving groundwater systems were conducted in the 1970s and 1980s. Most notable were transport studies involving the use of an *Aerobacter aerogenes* phage (9), phage T4 in a gravel aquifer in New Zealand (10), three unspecified phages, having *Escherichia coli* K12, *Serratia marcescens* and *Enterobacter cloacae* as their respective hosts, in a chalk aquifer in the United Kingdom (11,12), phages f2 and P22H5 in a karst aquifer in Greece (13), and MS2 in a sandy aquifer in Arizona (14). A protocol for using phages as groundwater tracers in granular aquifers is depicted in Figure 1.

Many of the bacteriophages commonly used as tracers in groundwater applications are coliphages, that is, they have *E. coli* as their host. Although there are many RNA- and DNA-containing coliphages (15), only a few (e.g., MS2, T4, T7, ϕX174, and f2) were used in tracer applications involving subsurface media. In addition to the aforementioned list of coliphage tracers, it is noted that the popular tracer phage, PRDI, which can also infect a strain of E. coli, has *Salmonella typhimurium* as its primary host. Numerous injection and recovery studies were conducted in the 1990s that involved comparing coliphage and conservative solute tracers in a variety of groundwater environments. The tracer test sites included sandy aquifers in Cape Cod, Massachusetts (16-18), and Ontario (19), highly fractured till in Ontario (20), fractured saprolyte in Tennessee (21), a floodplain aquifer in Montana (22), an alluvial aquifer in New Zealand (23,24), and a dune recharge site in the Netherlands (25). In addition to their widespread use with conservative tracers in injection and recovery experiments, coliphages are often used as indicators or tracers of fecal contamination in groundwater environments, including recent groundwater studies in the United States (26,27), Spain (28), Finland (29), and Israel (30). As noted by Edberg and coworkers (3 1) and Leclerc and coworkers (32), there are a number of problems associated with the uses of coliphages as tracers of groundwater contamination and the future of such uses remains unclear. A major problem is the lack of a strong relationship between bacteriophage recovery from groundwater and enteric gastroenteritis outbreaks attributed specifically to enteric viruses. A detailed discussion of the use of coliphages as indicators of human enteric viruses in groundwater is provided by Snowdon and Cliver (15).

Recently, various marine phages have been used with conservative solutes as groundwater tracers to understand better the geohydrology in karst (7), heterogeneous (periglacial) granular (7,33), and fractured-granite (33) aquifers in Switzerland. Unlike coliphages and phages of other bacterial inhabitants of mammalian intestinal tracts, marine phages are not a regulatory or water quality concern because their hosts are harmless. Also, the background concentration of marine phages in freshwater aquifers should be zero, even in groundwater contaminated by domestic or agriculture wastes. Comparisons of the transport characteristics of different marine phages with coliphages, indicate that at least one marine phage,

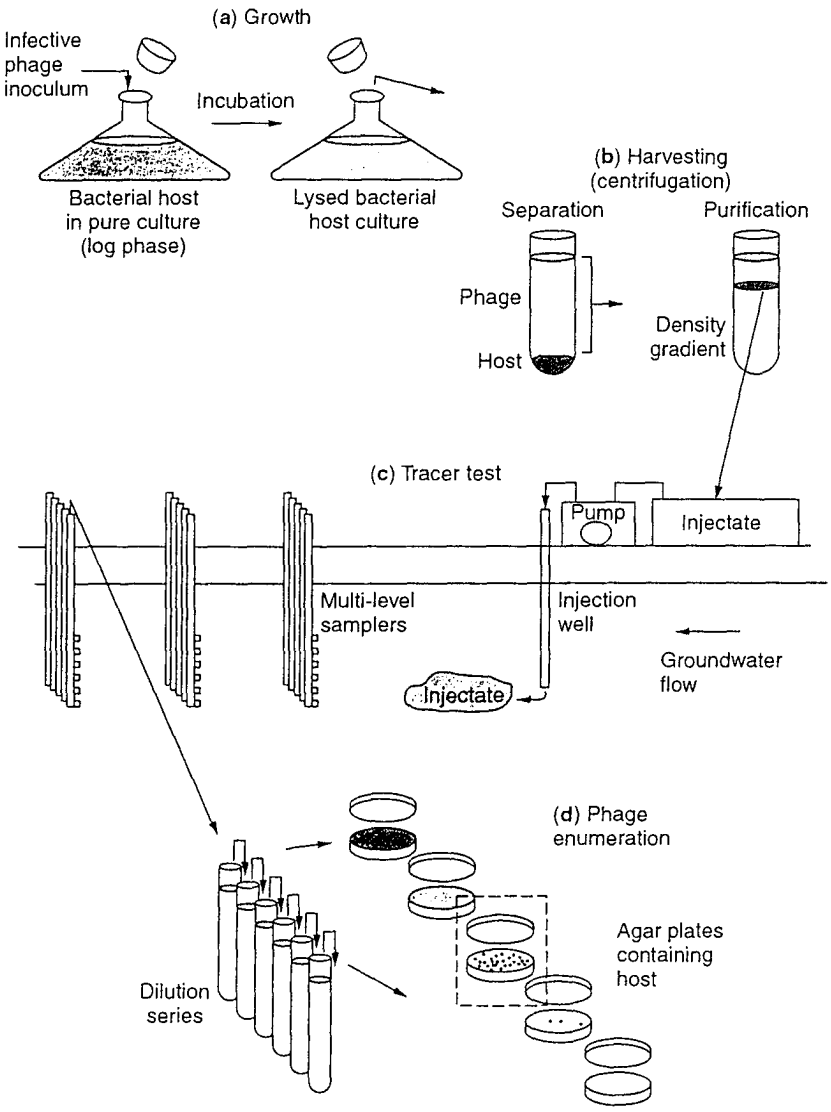


Figure 1. Protocol for the use of bacteria-specific viruses (bacteriophages) as tracers in injection and recovery tests in granular aquifer sediments: (a) Growth involves infecting the host bacterium during log phase growth in nutrient broth. (b) Purification of the phage involves initial separation of host cell debris from the new phage by centrifugation, followed by density gradient centrifugation of the supernatant. (c) Natural-gradient transport study involves a pulse injection of the purified phage and a conservative solute tracer upgradient from an array of multilevel samplers that are in rows perpendicular to the direction of groundwater flow. (d) Enumeration of phages in groundwater samples involves a dilution series and enumerating plaques (clearing zones) in agar plates containing a cloudy suspension of the bacteria] host.

H40, may have superior transport characteristics to the coliphage MS2 (33). Recent genetic studies show that marine phages are closely related to coliphages, although their life histories can be quite different (34). Although marine phages have not been widely used as groundwater tracers outside Switzerland, the authors anticipate that their use in injection and recovery experiments will spread. Conversely, phages commonly associated with contaminated freshwater environments were used to trace septic tank contamination in the Florida Keys into the marine environment (35).

All tracer experiments employing phages have the drawback that the standard plaque assays do not account for the total mass of the phage in the sample. This is because the phages are subject to an inactivation rate (rate of loss of host infectivity). Although the inactivation rate often is described as a simple function of temperature, there is increasing evidence that inactivation is more complicated and in some cases, loss of infectivity in groundwater systems may be poorly calculated by mean groundwater temperature. This is because the inactivation rates are subject to change, depending on whether the phage is attached to surfaces or unattached. Also, the intersurface forces that come into play when phages interact with solution-solid and water-air interfaces can cause inactivation. One method of assessing the true mass movement of phages in the aquifer involves radiolabeling the protein capsid with $S^{35}$ or genetic material therein with $P^{32}$ (17,18). However, the hazards and regulatory issues involving the use of radiolabeled phages restrict its use to a small number of controlled groundwater study sites.

**Bacteria**

The choice of bacteria in the first (nineteenth century) injection and recovery microbial tracer studies was made primarily because of their ease of detection in collected groundwater samples, rather than their transport or survival characteristics. The fact that the bacteria employed in the early studies formed brightly colored (red or yellow) colonies on solid media greatly facilitated differentiation of the tracer bacterium from the indigenous bacteria in the aquifer. There was a dearth of similar

injection and recovery experiments through the first seven decades of the twentieth century, although a number of microbial tracer studies focused on tracking bacterial indicators of fecal contamination downgradient from known sources (36,37). Such studies provide very limited information about subsurface microbial transport because of difficulties in defining the source. A notable exception involved a bacterial tracer study in which known quantities of specific pigment-producing bacteria, including *S. marcescens,* were introduced into an Alaskan aquifer and tracked downgradient (38).

A resurgence in injection and recovery tests involving bacterial tracers occurred in the 1970s and 1980s. A number of bacterial tracer studies published during that period employed antibiotic resistance as a mechanism to facilitate differentiation of the bacteria of interest from other bacteria in groundwater samples (39,40). More definitive labeling of bacteria with a DNA-specific fluorescent (fluorochrome) stain, that is, DAPI (4',6-diamidino-2-phenylindole) (41) and a radioisotope (p32) (42) facilitated bacte-rial tracer experiments in the late 1980s. Other fluorochromes possessing a variety of wavelengths of excitation and emission are available for labeling bacteria and were considered in tracer applications. However, unlike DAPI, (43,44), several of the more common fluorochromes, that is, ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide), acridine orange (3,6-bis(dimethylamino)acridinium chloride), and Hoechst 33,258 (2'-(4-bydroxylphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-l-H-benzi-midazole) can alter attachment behavior of bacteria in porous media (44), thus limiting their use in groundwater tracer applications.

In the 1990s, microbial tracer studies involved tracking nalidixic acid-resistant (45) and antibiotic-resistant (46) E. coli and antibiotic-resistant *Pseudomonas fluorescens* (47) through the subsurface. Also, in the 1990s, the first bacterial tracer tests were conducted in which bacteria that were labeled with a stable isotope ($C^{13}$) (48) and a vital fluorescent stain, 5-(and 6-)-carboxy-fluorescein diacetate, succinimidyl ester (CFDA/SE) (49), were injected into a sandy aquifer in Virginia. Also, a bacterial tracer was developed based on the ability of some naturally occurring and engineered bacteria to facilitate the formation of ice in small water droplets at approximately 5°C (50). One such ice nucleation active (INA) bacterium, *Pseudomonas syringae,* was employed as a readily identifiable bacterial tracer in a tracer experiment involving a shale-saprolyte aquifer in Tennessee (21). A discussion of methods to label bacteria apriori for subsequent groundwater tracer studies is provided by Harvey and Harms (51) and Deflaun and coworkers (52). Colwell and coworkers (53) discuss the advantages of using a cyanobacterium as an easily quantified particulate tracer in the terrestrial subsurface. Beeman and Suflita (54) describe the use, as tracers, of sulfate-reducing bacteria serendipitously present in drilling muds to help assess to what degree subsurface core sample are com-promised by the inadvertent introduction of nonindigenous microorganisms through drilling and recovery operations.

### Yeast and Protozoa

Uses of yeasts and protozoa in groundwater tracer applications have been limited. The large size of yeasts and protozoa may preclude their use in many aquifer studies because of problems relating to correspondingly high sedimentation (settling) rates. Although the first use of eukaryotic microorganisms as tracers in groundwater studies is not clear, Reichert (55) mentions the use of "yeast bacteria" in aquifer tracer studies conducted in the early 1900s. In 1975 and 1976, hydrologic studies were conducted in Texas in which 3 μm Baker's yeast *(Saccharomyces cerevisiae)* were employed as particulate groundwater tracers along with conservative halide (chloride, bromide, and iodide) tracers. The purpose was to delineate secondary permeability features within a heterogeneous granular aquifer interdispersed with clay and partly cemented with calcium carbonate (56). Baker's yeast had the advantages of being easily obtainable in large (kg) quantities and being easily identified and counted. The first use of a protozoan tracer in an injection and recovery experiment occurred in 1991. In order to better understand the transport behavior of groundwater protists, the nanoflagellate, *Spumella guttula* (Kent) was isolated from a sandy aquifer in Massachusetts, grown up in liquid media, labeled with the vital stain hydroethidine, and injected back into the aquifer along with bromide (57). A subsequent experiment was conducted in 1994, in which S. guttula was grown in aquifer sediments under low pH and carbon conditions, labeled with DAPI, and again injected into the aquifer with a conservative tracer. Although the smaller (2 to 3 μm), porous-media-grown nanoflagellates were advected through the sandy sediments (58), their delicate nature and the difficulties in obtaining large numbers limit their usefulness as groundwater tracers.

### Microspheres and Spores

Well-characterized, microbial-sized microspheres (minute beads consisting of organic polymers) were first employed as particulate surrogates for microbial groundwater tracers starting in the 1980s. In 1986, bacteria-sized (0.2, 0.5, 0.6, 0.7, 0.9, 1.4 μm) microspheres that differed in surface properties were injected into and recovered from a sandy aquifer in Cape Cod, Massachusetts to gain information about the physicochemical controls of subsurface microbial transport (Table 1; 41). Microspheres in the 0.8, 1.2, and 2.1 μm size class were also injected into and recovered from a fractured-granite aquifer at the Chalk River site in Ontario (42). A number of injection and recovery experiments employing virus-, bacteria-, and (or) protist-sized microspheres in a variety of geohydrologic environments were conducted in the 1990s. These tests included injections of both bacteria-sized (0.7 μm) (16,59) and protozoa-sized (57) (2 to 6 μm) microspheres at the Cape Cod site, bacteria-sized (0.2 to 1.0 μm) microspheres in a fractured-rock sites in Northern California and New Hampshire (60), bac-teria-sized microspheres in marine sediments (61), virus-sized (100 nm) microspheres in a weathered shale saprolyte in Tennessee (21), and protist-sized (2, 5, and 15 μm) microspheres in a heterogeneous granular aquifer in Idaho (62).

**Table 1. Use of Microbial-Sized Microsphere Tracers in Granular Aquifers (in Progress)**

| Factor aquifer | Microsphere | Size μm | Test Type | Test Distance[a] | RF[b] % | RF[c] | Ref. |
|---|---|---|---|---|---|---|---|
| Heterogeneity | | | | | | | |
| silt/sand/gravel/pebbles | Polystyrene | 2 | FG[d] | 1–4 | 0.2–0.5 | NC[e] | 62 |
| well-sorted sand | Carboxylated | 2 | NG[e] | 2–4 | 0.5–1.0 | NC[e] | 57 |
| well-sorted sand | Carboxylated | 0.7 | NG | 6 | 1.0–1.7[f] | NC[e] | 59 |
| Charge | | | | | | | |
| well-sorted sand | Uncharged latex | 0.5 | NG | 7 | 1.0 | 0.05 | 41 |
| well-sorted sand | Polyacrolein | 0.8 | NG | 7 | 1.3 | 3.11 | 41 |
| well-sorted sand | Carboxylated | 0.5 | NG | 7 | 1.4 | 0.04 | 41 |
| Size | | | | | | | |
| well-sorted sand | Carboxylated | 0.2 | NG | 7 | 1.4 | 0.01 | 41 |
| well-sorted sand | Carboxylated | 0.9 | NG | 7 | 1.4 | 0.06 | 41 |
| well-sorted sand | Carboxylated | 1.4 | NG | 7 | 1.1 | 0.12 | 41 |

[a] meters,
[b] RF is retardation factor (ratios of the respective velocities of the peak concentrations of the microorganisms and conservative solutes),
[c] RB is relative breakthrough, determined by dividing the numerical integration of the microspheres abundance versus time by that of the conservative tracer,
[d] NG is natural-gradient,
[e] NC = not calculated,
[e] FG is forced-gradient,
[f] Range of values correspond to the three different depths, that is, 8.8, 9.0, and 9.5 m below land surface.

Microbial-sized microspheres sometimes are injected into the subsurface to determine the degree by which the aquifer material, subsequently recovered from the same site, has been compromised by drilling and recovery activities. This microsphere tracer application involves determination of the number of log unit reductions between the abundance of microspheres added to the drilling fluid or borehole and the microsphere abundance in the recovered core material. Examples of this use of microspheres as subsurface tracers for quality-control purposes include studies involving deep aquifer sediments in South Carolina (63) and subsurface sediment and basalt in Idaho (64).

Various types of spores have been used as particulate tracers in groundwater systems. Many of these studies involved *Lycopodium clavatum* (clubmoss) spores that are typically approximately 30 μm and are stained apriori for easy identify-cation in groundwater samples. The first published use of *L. clavatum* spores as groundwater tracers occurred in 1926 (65). However, stained spores of *L. clavatum* have largely been restricted to hydrologic studies involving karst aquifers (66,67). This is because the relatively large sizes of *L. clavatum* spores render them a less attractive choice in many fractured-rock and granular aquifers. Kass and Reichert (68) describe methodologies for using spores as groundwater tracers, including preparation, sampling, and quantification. Endospores of the bacterium, *Bacillus subtilis* were used in several injection and recovery studies involving heterogeneous granular aquifers in New Zealand (24). The small (<1 μm) size and high resistance to environmental conditions make bacterial endospores better candidates as groundwater tracers in many granular and fractured groundwater systems.

## HYDROLOGIC STUDIES

The search for definitive, quantifiable particulate tracers to understand better the hydrology of different types of aquifers provided the impetus for the first uses of microorganisms as groundwater tracers. It should be noted that particulate and solute tracers can exhibit very different transport behaviors in the same aquifer. Many of the hydrologic studies employing microbial tracers that were conducted during the last few decades used both particulate and conservative solutes. In the process of assessing and comparing the breakthrough curves of both microbial and solute tracers, important information has been acquired, not only about the hydrology of aquifers, but about how microorganisms being transported through different groundwater environments are affected by chemical and physical heterogeneities.

Table 2 lists the retardation factors (ratio of the velocity of the microorganisms to that of the conservative tracers) for microbes being advected downgradient in injection and recovery tests. Results are compared for a number of different types of subsurface media. The retardation of microorganisms relative to a conserv-ative tracer is a very complex phenomenon, but is strongly af-fected by the degrees of physical and chemical heterogeneity. In fractured media, the peak concentration of microorganisms ap-pearing downgradient often significantly precedes that of a con-servative tracer. For example, it is reported that the velocities a associated with peak concentrations of microbial tracers exceeded those of a conservative tracer by two or more factors of ten in aquifers characterized by high heterogeneity and fracture-flow, that is, weathered-shale saprolyte (21) or clay-rich till (69). This leads to calculated retardation factors for microorganisms that are substantially less than one. The specific mechanism for

**Table 2. Retardation in Transport of Microorganisms Relative to Conservative Solutes for In situ Tracer Experiments Involving Different Types of Subsurface Media**

| Media Type | Microorganism(s) | | Test | | RF[a] | Ref. |
|---|---|---|---|---|---|---|
| | Species | Type | Type | Distance | | |
| Fractured | | | | | | |
| Shale saprolyte (Tennessee) | MS2, PRD1 | phage | NG[b] | 35 m | 0.002 | 21 |
| Clay-rich till (Ontario) | MS2 & PRD1 | phage | NG | 4 m | 0.01 | 69 |
| Granite (Ontario) | *Escherichia coli* | bacterium | FG[c] | 13 m | 0.1 | 42 |
| Granite (Switzerland) | Hb, H40, MS2 | phages | FG | 1.2 m | 0.4–0.7 | 33 |
| Layered basalt (Hawaii) | *Bacillus* sp | bacterium | FG | 27 m | 0.6 | 5 |
| Granular, highly heterogeneous | | | | | | |
| Periglacial gravel/cobbles (Switzerland) | H4, H6, H40 | marine phages | NG | 10–64 m | 0.2–0.9 | 33 |
| Sand/gravel/clay/carbonates (Texas) | *Saccharomyces cerevisia* | yeast | FG | 1.5 m | 0.7 | 56 |
| Alluvial gravel (New Zealand) | *Bacillus subtilis* endospores | bacterium | NG | 20–90 m | 0.9 | 70 |
| Karst | | | | | | |
| Jura limestone (Switzerland) | H40 & H6 | marine phages | NG | 2.5 km | 1.0 | 7 |
| Lurbach System karst (Austria) | P22H5 | phage | NG | 5 km | 1.0 | 6 |
| Granular, Relatively Homogeneous | | | | | | |
| Well-sorted sand & gravel | Indigenous community | bacteria | FG | 1.7 m | 1.0 | 41 |
| (Massachusetts) | Indigenous community | bacteria | NG | 6.7 | 1.0 | 70 |
| | PRD1 | phage | NG | 13 | 1.0 | 16 |
| | *Spumella guttula* (Kent) | nanoflagellate | NG | 1.0 | 1.0[e] | 58 |
| Granular, unsaturated | | | | | | |
| Tujunga loamy sand (California) | MS-2 | phage | UF[d] | 0.3 m | 254 | 71 |

[a]Retardation factor (ratios of the respective velocities of the peak concentrations of the microorganisms and conservative solutes).
[b]NG is natural-gradient,
[c]FG is forced-gradient,
[d]UF is unsaturated flow.
[e]Flagellates grown in liquid broth exhibited retardation factors of up to 4.7 (31) at the same field-site location.

apparently faster transport of the peak concentrations of microorganisms relative to those of conservative tracers remains unclear, although volume/pore-size exclusion of the microorganisms, hydrodynamic retardation of the conservative solute, and immobilization of the trailing microbes can contribute to this phenomenon. Studies performed in highly heterogeneous granular aquifers in New Zealand and Switzerland indicate that the appearance of the peak abundance of microbial tracers can substantially precede that of conservative tracers, although substantially less so than in aquifers dominated by fracture flow.

A number of studies involving relatively homogeneous well-sorted granular media in Massachusetts suggest that the transport of viral-, bacterial-, and protozoan-tracers can often occur concomitantly with that of the conservative tracer (16,41,59,71), that is, the retardation factor is unity. This suggests that the microbial and conservative tracers are taking the same flow paths and accessing the same pore volumes. Studies involving karstic aquifers indicate that peak concentrations of microbial tracers and conservative solutes can also appear coincidently (6,7). However, such observations involving karst undoubtedly depend on the physical nature of the aquifer. Finally, substantial retardation of microorganisms advecting through granular media may be expected under conditions of unsaturated flow (72). This is because microorganisms, unlike conservative solutes, become trapped at air-water interfaces that move much more slowly than the bulk flow of water.

Microspheres were extensively used, in lieu of and in addition to microbial tracers, to gain information concerning the hydrology of different types of aquifers. Data concerning selected studies involving the injection and recovery of microbial-sized microsphere tracers that have resulted in information about how microorganisms move through granular aquifers are summarized in Table 1. Results of these studies indicate the roles of heterogeneity in aquifer structure, of microbial size, and of microbial surface charge upon the retardation and relative breakthrough/immobilization of microorganisms, as they ' are advected downgradient. Microspheres with similar surface chemistries and buoyant densities, but different sizes, were important in determining the effect of microbial size during transport through granular (41) and highly fractured (60,73) aquifers. Microspheres were also useful in determining the suitability of applying the clean-bed colloid-filtration theory (74) in multi component models describing microbial transport in a well-sorted sandy aquifer (41,71) and in determining whether or not 2 to 15 μm sized bacteria-containing encapsulations could be efficiently dispersed in a heterogeneous granular aquifer (41).

## PUBLIC HEALTH STUDIES

In the United States, subsurface transport of pathogens continues to be responsible for a majority of the outbreaks of waterborne disease. The interested reader is referred

to Macler and Merkle (75) for discussions of what is currently known about groundwater pathogens and their control. Valuable information was gathered about how pathogens behave in the subsurface by using microbial tracers, including the use of nonpathogenic surrogates in groundwater injection and recovery experiments. More specifically, microbial tracers have been useful tools for delineating the likelihood of pathogen transport pathways from known sources of subsurface microbial contamination and have provided a better delineation of how pathogens move in different types of media. Microbial tracer information has been valuable to regulators, environmental engineers, public health officials, and environmental microbiologists.

Future experiments should focus on the use of tracer microorganisms to assess the vulnerability of various aquifers to microbial contamination. Of particular concern is microbial contamination resulting from ongoing and planned aquifer storage and recovery, deep well injection of treated domestic wastewater, artificial recharge, bank filtration, and deployment of decentralized waster water treatment systems (e.g., septic tanks). The utility of a microbial tracer for aquifer vulnerability studies depend, in part, on how well the tracer represents the survival and transport characteristics of the pathogens of concern in groundwater. Macler and Merkle (75) list the contaminant groundwater viruses of concern as Coxsackie, Echo, Norwalk, Hepatitis A & E, Rota, Enteric adeno, Calici, and Astro viruses. They also list the bacterial pathogens of concern as E. *coli, Salmonella spp., Shigella spp., Campylobacter jejuni, Yersinia spp., Legionella spp., Vibrio cholera* and the protozoan pathogens as the obligate parasites *Cryptosporidium parvum* and *Giardia lamblia.* However, it would be difficult, at best, to find a microbial tracer that represents the transport and survival of more than a few of the aforementioned pathogens.

Phages have often been the microbial tracer of choice, largely because they are harmless, easy to assay using standard plaque assays, and exhibit good survival characteristics (3). Phages are found in a number of habitats, including marine and freshwater environments, wastewater, soils, plants, animals, and food (7). It is clear that phages are an important and frequently abundant component of a number of aquatic ecosystems (76,77). For some marine systems, phage abundances can be as high as $10^7$ to $10^8$ per mL (78). However, phage tracers can be differentiated easily from the often abundant indigenous phages in aqueous systems because of their high degree of host specificity, often involving a single bacteria] host. Their small size (10 to 200 nm) also contributes to their suitability as a tracer because it results in negligible settling, good penetration into smaller porosity, and a low propensity for clogging of granular media. Although a number of phages, particularly coliphages, were used as tracer viruses in public health studies, a number of recent studies focused specifically on PRD1 and (or) MS2. Schijven (79,80) describes the extensive series of experiments and modeling efforts concerning the use of PRD1 and MS2 to assess the efficacy of groundwater environments in the Netherlands to remove harmful viruses. Unfortunately, important questions remain about how well PRD1 and MS2 represent the pathogenic viruses of interest in groundwater applications. At the time of this writing, detailed in situ studies were yet to be conducted to determine the suitability of many other phages as groundwater tracers or as surrogates for other viruses.

## BIOAUGMENTATION/BIORESTORATION STUDIES

Recently, experiments were conducted in which microorganisms were added to subsurface media and used as tracers to gain useful information for the growing field of in situ bioremediation. Bioaugmentation involves the addition to the contaminated subsurface of specialized or genetically engineered microorganisms (GEMs) to affect a measurable degree of biodegradation. The majority of studies supporting bioaugmentation involved laboratory microcosms. This is, in part, because regulatory concerns have restricted the uses of GEMs in the field. However, critical information needed to predict the potential of GEMs to disperse and survive in contaminated subsurface environments must come from in situ experiments. This is because many of the complex and interrelated processes operative on the field-scale are not represented well in lab-scale studies. One of the first microbial "tracer" applications in support of future biorestoration procedures involved the injection of *Pseudomonas* sp. strain B13, a 3-chlorobenzoate metabolizing bacterium, into the aquifer in Cape Cod, MA. The bacterium was subsequently recovered from aquifer sediments 14 months later (81) suggesting that the survival time in an aquifer for a nonindigenous, genetically altered bacterium may be long enough to enhance aquifer remediation, at least under some circumstances. Although 3-chlorobenzoate was not present around the zone of injection, the genes required to affect degradation of that compound served as a key to its identification and quantification in aquifer samples. In a subsequent in situ bioremediation study, the bacterium *Pseudomonas stutzeri* strain KC that degrades carbon tetrachloride (CT) under denitrifying conditions was injected into a granular, CT- and nitrate-contaminated aquifer in Michigan (82). The bacterium not only served as its own microbial tracer, based on its DNA sequences and tendency to form distinctive "fried-egg" shaped colonies on solid media, it substantively enhanced the degradation of CT within the aquifer.

Another "tracer" application of bacteria for contaminated subsurface environments involves the use of specific GEMs as "sentinels" or indicators of whether or not specific contaminants are present and how the contaminants are distributed. One study involved a controlled, intermediate-scale field release of a bioluminescent bacterium *P. fluorescens* Hk44 in a vadose zone contaminated with polyaromatic hydrocarbons (PAHs). The bacterium harbored an introduced *lux* gene fused with a naphthalene-degradative pathway, allowing the bacterium to bioluminesce in the presence of naphthalene (83). The bioluminescence at depth then was measured by employing fiber-optic-based biosensor devices. The use of *lux*-based bioreporter bacteria such as *P. fluorescens* Hk44 as tracers for subsurface contaminants is a promising new tool in aquifer biorestoration.

Another recent subsurface bioaugmentation study involving the addition of a GEM included the addition to atrazine-contaminated soil of killed, stabilized E. coli genetically engineered to overproduce atrazine chlorohydrolase (84). Results supported the tractability for using bacteria engineered with "overexpressing" catabolic genes to increase significantly in situ biodegradation rates. However, many technical problems regarding the use of GEMs in subsurface bioaugmentation lie ahead. Recently, the field-usage of GEMs was permitted at a number of locations. Consequently, future additions of GEMs to contaminated aquifers and vadose zones are likely to be forthcoming in order to gain more information about the feasibility of using different recombinant bacteria to enhance biodegradation in a variety of subsurface environments. In these types of experiments, the GEMs can serve both as microbial tracers, because their engineered DNA sequences can be traced in space and time, and as agents of bioremediation.

## CONCLUSION

In conclusion, microorganisms and microbial-sized microspheres are used as tracers in groundwater-related studies in many ways. The first injection and recovery studies in which microorganisms were used to delineate groundwater flow in karst have evolved into much more complex experiments involving both conservative and particulate tracers and well-instrumented sites in many different geohydrologic environments. The use of more sophisticated labeling methods, such as the use of recombinant DNA, is allowing for more efficient differentiation and quantification of microorganisms introduced into freshwater aquifers. In turn, the increasing number and technical sophistication of injection and recovery studies employing microbial tracers will further enhance our knowledge of groundwater systems, particularly karstic, fractured rock, and heterogeneous granular aquifers, and of the potential fate of pathogens in different types of groundwater environments. Studies that employ microorganisms as tracers also facilitate new bioaugmentation technologies that will be used in future aquifer restoration projects.

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