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Transport Behavior of Groundwater Protozoa and Protozoan-Sized Microspheres in Sandy Aquifer Sediments

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Transport behaviors of unidentified flagellated protozoa (flagellates) and flagellate-sized carboxylated microspheres in sandy, organically contaminated aquifer sediments were investigated in a small-scale (1 to 4-m travel distance) natural-gradient tracer test on Cape Cod and in flow-through columns packed with sieved (0.5- to 1.0-mm grain size) aquifer sediments. The minute (average in situ cell size, 2 to 3 μm) flagellates, which are relatively abundant in the Cape Cod aquifer, were isolated from core samples, grown in a grass extract medium, labeled with hydroethidine (a vital eukaryotic stain), and coinjected into aquifer sediments along with bromide, a conservative tracer. The 2-μm flagellates appeared to be near the optimal size for transport, judging from flowthrough column experiments involving a polydispersed (0.7 to 6.2 μm in diameter) suspension of carboxylated microspheres. However, immobilization within the aquifer sediments accounted for a log unit reduction over the first meter of travel compared with a log unit reduction over the first 10 m of travel for indigenous, free-living groundwater bacteria in earlier tests. High rates of flagellate immobilization in the presence of aquifer sediments also was observed in the laboratory. However, immobilization rates for the laboratory-grown flagellates (initially 4 to 5 μm) injected into the aquifer were not constant and decreased noticeably with increasing time and distance of travel. The decrease in propensity for grain surfaces was accompanied by a decrease in cell size, as the flagellates presumably readapted to aquifer conditions. Retardation and apparent dispersion were generally at least twofold greater than those observed earlier for indigenous groundwater bacteria but were much closer to those observed for highly surface active carboxylated latex microspheres. Field and laboratory results suggest that 2-μm carboxylated microspheres may be useful as analogs in investigating several abiotic aspects of flagellate transport behavior in groundwater.

Although protozoa are now known to be common in both shallow (3, 19, 35, 38) and deep (over 200 m below land surface) (36) groundwater environments, little is known about their transport behavior. Important questions relating to the transport behavior of protozoa in groundwater environments involve the ability of relatively resistant protozoan pathogens (e.g., *Cryptosporidium* oocysts) to move through and contaminate drinking water aquifers, the ability of protozoa to move to contaminated areas of aquifers that are undergoing bioremediation, the influence of protozoan mobility on the spatial variability of eukaryotic community diversity within an aquifer, and the role of protozoan mobility in predation of groundwater bacteria. Increasing attention is being focused upon protozoan mobility in organically contaminated aquifers (15), where elevated numbers of protozoa (10^4 to $10^5 \cdot$ g of dry weight^{-1}) (23, 28, 37) are thought to be a potential factor in the fate of organic contaminants.

An aquifer contaminated by a 5-km-long plume of dilute, treated sewage from a trickling-filter treatment facility at Otis Air Base, Mass., served as the field site of our study. The site is the focus of a long-term collaborative study among the University of New Hampshire, the U.S. Geological Survey (USGS), and the Natural History Museum (London, England) involving the distribution, ecology, community structure, and potential remediative role of groundwater protozoa. Relative to more pristine zones of the aquifer, the contaminant plume harbors a large (up to $10^5 \cdot$ g of dry weight^{-1}) (23) and diverse population of cyst-forming, flagellated protozoa (flagellates). This population includes new and previously described species belonging to the genera *Bodo*, *Cercomonas*, *Cryptaulax*, *Cyathomonas*, *Goniomonas*, and *Spumella* (31). Factor analysis suggests that there may be some correlation between abundance of flagellates and numbers of relatively mobile free-living bacteria within the contaminant plume (24). The flagellate population includes genera commonly found in sewage and biological filters (e.g., *Bodo* [6]). However, there is a dearth of information on the makeup of the protozoan community in down-gradient areas of the contaminated zone. Consequently, comparisons between the extents of down-gradient travel of a "marker" species of flagellate and a near-conservative plume constituent (e.g., boron) would be difficult to interpret from their distributions within the plume.

The purpose of our study was to develop a better understanding of flagellate transport behavior in sandy aquifer sediments by using small-scale tracer tests. This was done by comparing the advective mobility of unidentified groundwater flagellates with that of conservative (bromide) and particulate (fluorescent microsphere) tracers in flowthrough columns (0.6-m travel distance) and in the field (1- to 4-m travel distance). Our first objective was to compare the degree of in situ flagellate immobilization, apparent dispersion, and retardation with that observed earlier (12, 13, 16) for free-living groundwater bacteria that may be serving as a protozoan food source. A second objective was to examine the importance of straining as an impediment to their advective movement. This was accomplished by comparing immobilization rates in sieved (0.5 to 1.0 mm) sediments (where straining would not be expected to occur) with those observed in undisturbed sediments. A third

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TABLE 1. Parameters for the injection of flagellates and microspheres into sandy aquifer sediments at the Cape Cod study site

Test	Well no.	Injection				Preinjection chemistry			Depth		
		Date	Vol (liters)	[Br^-] (mg/liter)	Duration (h)	TDS (mg/liter)	pH	Temp (°C)	Below surface (m)	Altitude (m)	Designation
Microspheres	M7-15	16 June	203	150	2.3	190	5.7	12	9.3	11.7	Orange
									9.6	11.4	Gray
Flagellates	M4-15	1 July	100	165	1.2	ND[a]	ND	ND	9.0	12.0	Gray
									9.3	11.7	Yellow

[a] ND, not determined.

objective was to assess the suitability of microspheres as abiotic analogs in future investigations involving the physical aspects of flagellate transport behavior. The latter study involved comparison of flagellate and microsphere transport behavior. In addition, the utility of polydispersed suspensions of microbe-sized microspheres to determine optimal flagellate size for transport was evaluated.

MATERIALS AND METHODS

Study site. The field site is within an unconfined, sandy, glacial outwash aquifer in Falmouth, Mass. (western Cape Cod), which has been contaminated by over 50 years of disposal of secondarily treated sewage onto rapid infiltration sand beds. The 5-km-long contaminant plume, from which the flagellates were isolated and in which the tracer test was run, is characterized by elevated temperature, specific conductance, and dissolved organic carbon (up to 18°C; 450 $\mu S \cdot cm^{-1}$, 4 $mg \cdot liter^{-1}$) relative to uncontaminated zones in the aquifer (10°C; <80 $S \cdot cm^{-1}$, <1 $mg \cdot liter^{-1}$). The aquifer sediments are composed largely of quartz and feldspar, with very little (<1%) clay. Hydraulic conductivity, average porosity, and mean grain size in the area of the transport experiment are ~0.1 $cm \cdot s^{-1}$ (18), 0.35, and ~0.59 mm, respectively (25). The average groundwater velocity in the area of the test is ~0.5 $m \cdot day^{-1}$ (26).

Protozoa. Flagellates were isolated from aquifer sediments taken aseptically from within the contaminant plume at 2 to 3 m below the water table at a USGS well site (F383) located near the transport test site. The sediments were taken without the use of drilling fluids, using a wireline piston-type coring device (43) in conjunction with a hollow-stem auger drill (11). For the field tracer (groundwater injection and recovery) experiments, flagellates were cultured in Fernbach flasks with Cerophyl-Prescott's Infusion medium (32), which caused them to grow larger (4 to 5 μm in diameter) than what is typically observed in situ (2 to 3 μm). The flagellates were then labeled with hydroethidine (HE; Polysciences), which is a vital eukaryotic stain produced by chemical reduction of the fluorochrome ethidium bromide (9). This was accomplished by adding 2 ml of staining solution (100 mg of HE in 16.4 ml of *N*,*N*-dimethylacetimide) per liter to 7-day cultures for 10 min. The HE-stained flagellates were kept in the dark at 4°C for 1 h and examined microscopically to ensure structural integrity prior to injection into the aquifer. The ability of HE-stained flagellates to survive for several weeks in groundwater was assessed by monitoring structural integrity and fluorescence intensity for a stained population in an area of the aquifer immediately adjacent to the point of injection and in laboratory microcosms.

Field tracer tests. The natural-gradient tracer (injection and recovery) experiments were performed within an area of the aquifer where previous investigations had been conducted involving subsurface transport behavior of indigenous groundwater bacteria (12, 13, 16). The present tests used small (4-m travel distance) areas of a large (20 by 220 m) array of 15-port multilevel samplers (MLS) as described by LeBlanc et al. (26). The MLS are constructed of 6.5-mm (diameter) polyethylene tubes to allow discrete sampling at depth (40). The use of MLS allows the tracking of injectate constituents (bromide and flagellates or bromide and microspheres) through undisturbed aquifer sediments within a three-dimensional grid. Bromide was used as a nonreactive tracer in both tests to establish the direction and extent of movement due to advection and the degree of chemical diffusion and hydrodynamic dispersion.

The first tracer test (June 1991) involved adding 2-μm carboxylated microspheres ($724 \pm 55 \cdot ml^{-1}$) and bromide (150 $mg \cdot liter^{-1}$)-labeled groundwater (203 liters) slowly to the aquifer at 11.7 and 11.4 m above sea level (9.3 and 9.6 m below land surface, respectively). Each constituent was then monitored as it moved with the natural flow of groundwater past MLS that were down gradient and in the path of the injectate cloud. The injection was made at well M7-15, which was the site of an earlier injection and recovery experiment involving the transport behavior of groundwater bacteria (16) and PRD-1 bacteriophage (1). Prior to injection, groundwater was collected from the two injection depths and used to make up the injectate in order to minimize chemical changes along the flow path of the injectate cloud. Preinjection groundwater chemistry and injection test parameters are given in Table 1. Dimensionless concentration histories for bromide and microspheres at 2.0 and 3.7 m down gradient from the point of injection were obtained by daily sampling from appropriate depths at wells M8-15 and M9-15, respectively.

Because of potential interference caused by the presence of brightly fluorescing microspheres upon detection of faintly fluorescing HE-labeled flagellates, in situ transport behavior of the latter was assessed in a separate tracer experiment (Table 1). The second tracer test (July 1991) involved adding 100 liters of groundwater containing HE-labeled flagellates ($2.2 \times 10^4 \cdot ml^{-1}$) and bromide ($165$ $mg \cdot liter^{-1}$) slowly to the aquifer at 12.0 and 11.7 m above sea level (9.0 and 9.3 m below land surface, respectively) at well M4-15, which is ~6 m upgradient from the site of the first test. It had been previously determined that the chosen bromide concentration did not adversely affect the survival of the stained flagellates over the time frame of the experiment. In order to capture breakthrough along the first 4 m of travel, groundwater samples (500 ml) were taken daily from MLS M4A-15, M5A-15, and M6-15. The sampling points were located along the trajectory of the injectate cloud at 1, 2.8, and 3.6 m down gradient from the points of injection.

Bromide was analyzed by using a specific ion electrode. Labeled flagellates and microspheres were both enumerated by epifluorescence microscopy. For protozoan enumerations, samples of groundwater were fixed (1% [wt/vol] final concentration) with glutaraldehyde that had been buffered in 0.001 M cacodylate (pH 7) and held in the dark at 4°C until processed. Appropriate volumes (50 to 250 ml) of sample were filtered onto 25-mm (diameter), 0.8-μm (pore size), black polycarbonate filters (Nuclepore Corp.). Backing filters (0.45-μm pore size; Gelman) were used to evenly distribute the vacuum. A transmembrane pressure of <0.3 atm was used for all samples to avoid lysis of the flagellates. In general, the groundwater flagellates do not remain intact with higher (0.8 atm) transmembrane pressures recommended in the membrane filter procedure for bacterial enumeration (20). The tendency to lyse was also reduced by the apriori fixation with glutaraldehyde. Enumerations were made under incident UV light, using a Nikon Optiphot epifluorescence microscope that was fitted with a UV-2A optical package (400-nm dichroic mirror, 330- to 380-nm excitation filter, and 420-nm barrier filter) by a scanning procedure described by Bunn (5). Standard deviations for replicate counts are generally ≤30% of the mean. Microspheres (type BB; Polysciences, Warrington, Pa.) were counted with a precision of ±10%, using UV excitation (340 to 380 nm) and a Leitz Dialux 20 microscope, fitted for epifluorescence as described by Harvey et al. (17).

Calculation of field transport parameters. Flagellate transport was evaluated for each of the down-gradient sampling points from observed concentration histories and from the following parameters: maximum dimensionless concentration, apparent longitudinal dispersion, retardation, relative breakthrough, and collision efficiency factor. The maximum dimensionless concentration, $(C/C_0)_{max}$, was calculated as the ratio of the highest flagellate concentration observed in samples collected down gradient to that present in the 100 liters of injectate. The apparent longitudinal dispersion (A_L) was calculated by the following relationship (12):

$$A_L = \frac{x_1(\Delta t/t_{peak})^2}{16 \ln 2}$$

where x_1 is the distance from the point of injection, Δt is the duration of breakthrough when $C(t) > 1/2$ peak concentration, and t_{peak} is the time to peak concentration. A_L values calculated for the flagellates are only first approximations because their concentration histories do not conform to the assumed classical Gaussian-shaped breakthrough curves upon which the above equation is based. Retardation was calculated as the ratio of time required for the arrival of the center of mass for the unattached flagellates to time to center of mass for bromide. The centers of masses for bromide and flagellates appearing at down-gradient samplers were determined by numerical integration of the respective concentration histories. Relative breakthrough (RB) was calculated as the integral of dimensionless concentration history normalized to that of bromide (13):

$$\mathrm{RB} = \int_{t_0}^{t_f} \frac{C(t)}{C_0} dt \div \int_{t_0}^{t_f} \frac{\mathrm{Br}^-(t)}{\mathrm{Br}_0^-} dt$$

where C_0 and Br_0^- are flagellate and bromide concentrations in the injectate, $C(t)$ and $\mathrm{Br}^-(t)$ are concentrations at time t, t_0 is the time of injection, and t_f is the elapsed time from the beginning of injection until the last sample was taken (45 days). The collision efficiency factor, α, which represents the physicochemical factors that determine immobilization of the flagellates, was calculated by using the following equation (12):

$$\alpha = \frac{d[[1-2(A_L/x_1)\ln(\mathrm{RB})]^2 - 1]}{6(1-\theta)\eta A_L}$$

where d is the median grain size (0.59 cm), x_1 is the travel distance to the MLS, θ is the porosity (0.35), and η is the single collector efficiency. The last parameter is the rate at which flagellates strike a single sand grain divided by the rate at which the flagellates move toward the grain and represents the physical factors determining collision. Its value was calculated by the following equation (41):

$$\eta = 0.9\left(\frac{kT}{\mu d_p d v}\right)^{2/3} + 1.5(d_p/d)^2 + \frac{(\rho_p - \rho)g{d_p}^2}{18\mu v}$$

where k is the Boltzman constant, T is the groundwater temperature, μ is the groundwater viscosity, d_p is the flagellate diameter, ρ is the groundwater density, ρ_p is the buoyant density of the flagellates (assumed to be ~1.05), g is the gravitational constant, and v is the groundwater velocity (calculated from arrival time for peak bromide concentration). This expression ignores close-approach effects and was used in lieu of the more rigorous relationship proposed by Rajagopalan and Tien (33), so that values of α could be compared with those calculated for bacteria in earlier experiments at the Cape Cod site (12). Values of collector efficiency calculated by the relationship of Rajagopalan and Tien (33) would be about fivefold higher than those calculated with the above equation.

Column experiments. The transport behavior of groundwater flagellates under more controlled conditions was assessed in flowthrough columns packed with sieved aquifer sediments. Chromaflex (Kontes, Vineland, N.J.) chromatographic glass columns (0.6 m by 4.8-cm inside diameter) were used with flow adapter-end caps. Polyethylene bed supports in the end caps were replaced with stainless-steel wire mesh (mesh sizes, 40 and 325; Small Parts, Inc., Miami, Fla.), which is less hydrophobic and should have a lesser tendency to sorb organic colloids. The sediments, which were collected from the Cape Cod site with an auger drill to bring the cuttings to the surface, were sieved and dry packed, using the method of Johnson (21) to achieve near-uniform packing. The method involved the sequential packing of oven-dried (37°C), 0.5- to 1.0-mm (grain size) sediments in 20 individual homogenized layers of ~80 g each, followed by careful inspection to ensure reasonable uniformity of packing. To preclude formation of air pockets during saturation of the column, air within the unsaturated sand was initially displaced by CO_2 (90 min at 0.3 $m^3 \cdot h^{-1}$), which was then displaced by 1.5 liters of 0.005 M $CaSO_4$ (to enhance CO_2 solubility) and, finally, by distilled water.

The columns were run under constant head in an upflow mode (10° from horizontal) in an environmentally controlled (10°C) chamber. The injectate (150 mg of bromide · $liter^{-1}$ and 6.3×10^4 to 7.6×10^4 HE-stained flagellates · ml^{-1}) was supplied to the column in a continuous fashion, using a 4-liter reservoir bottle, which siphoned into a tightly stoppered 1-liter Erlenmeyer flask set up to provide constant head. Flagellates were fed to the column for the equivalent of 1 pore volume. Both vessels were continuously stirred to ensure uniformity in flagellate abundance in the column influent. Constant head was achieved by fixing the location of the influent feed tube to the level of the liquid-air interface within the Erlenmeyer flask containing 0.40 liter of injectate. The injectate was fed to the columns with 3.1-mm (inside diameter) tygon tubing. The flow rate was controlled by a 30-cm-long segment of 0.51-mm (inside diameter) AutoAnalyzer tubing (Cole Parmer, Chicago, Ill.), which was inserted into the tygon influent tubing by twist connectors. Tee valves between the removable AutoAnalyzer tubing and the column allowed influent sampling during the course of the experiment and presaturation purge of air by CO_2. Effluent from the column was collected in borosilicate scintillation vials placed in an Eldex (San Carlos, Calif.) model U1A fraction collector that was set to advance at regular intervals. Flow rate through the column was monitored by measuring the volume collected during each timed (8-min) interval. Flagellate and bromide abundances were assessed by the methods described for the field experiments. Concentration histories of stained flagellates in the column effluent were compared with that of bromide after normalizing constituent concentrations to their respective injectate concentrations.

A second column investigation was conducted to assess the relative mobility of various size classes (0.7, 1.7, 2.8, and 6.2 μm) of carboxylated microspheres (1.05 g · ml^{-1}, specific gravity) within the size range of groundwater protozoa. The setup for this experiment was essentially the same as that described above for the flow column experiment involving flagellate transport and that described for an earlier experiment involving the role of physical heterogeneity in the relative mobilities of different-sized microspheres in the 0.4- to 4.8-μm range (16). The flow rate, temperature, and column inclination were ~2 ml · min^{-1}, 10°C, and 10° to the horizontal (upflow mode), respectively (27).

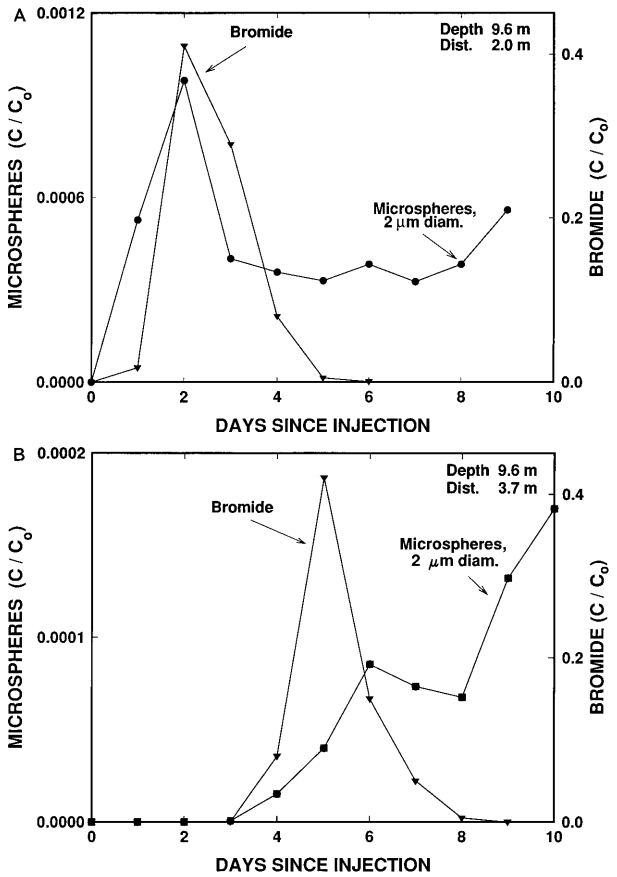

FIG. 1. Dimensionless concentration histories for 2-μm (diameter) carboxylated microspheres and bromide in sandy aquifer sediments in a natural-gradient transport experiment at USGS MLS M7-15. Breakthrough curves are for 2 (A) and 3.7 (B) m down gradient from the point of injection at 9.6 m below land surfaces.

RESULTS

Field. Dimensionless concentration histories for bromide and flagellate-sized (2-μm), carboxylated microspheres in the June 1991 tracer test are shown in Fig. 1 for the 9.6-m depth. Breakthrough curves for MLS M8-15 (2.0 m down gradient) and M9-15 (3.7 m down gradient) are depicted in Fig. 1A and B, respectively. At 2.0-m down gradient from the point of injection, the peak in microsphere abundance arrived coincidentally with that of bromide. However, at least half of the microspheres in the sampled portion of the breakthrough appeared after the bromide cloud had already passed. The duration of sampling was not sufficient to accurately determine a retardation or dispersion factor for the microspheres at either sampling point. However, it appears that the microspheres were subject to substantial apparent dispersion, judging from the protracted tail of the breakthrough curve at 2.0 m down gradient (Fig. 1A). Also, the 10-day concentration histories suggest that the microspheres were retarded by a factor of at least 2 after 3.7 m of travel (Fig. 1B), similar to that observed for the flagellates after 3.6 m of travel (Table 2). Microspheres appearing at the 9.3-m depth of MLS M8-15 and M9-15 were

TABLE 2. Transport parameters for bromide and HE-stained flagellates in a natural-gradient test (Cape Cod aquifer)

Distance (m)	Well	Depth (mbs)[b]	Groundwater velocity (m · day^{-1})	Apparent dispersion (cm)		Retardation factor[c]	Immobilization	
				Bromide	Flagellates		% Retained	α (10^{-2})[d]
1.0	M4A-15	9.0	0.50	9.0	21.0	6.1	83	3.8
		9.3	0.50	7.2	19.9	3.3	91	6.9
2.8	M5A-15	9.0	0.47	2.8	11.4	2.6	97	2.6
		9.3	0.70	7.8	11.2	2.3	98	4.2
3.6	M6-15	9.0	0.45	3.4	ND[e]	2.7	98	ND
		9.3	0.33	1.8	ND	2.1	99	ND

[a] Down-gradient distance from the point of injection (USGS well M4-15).
[b] mbs, meters below the land surface.
[c] Calculated as the ratio of bromide to flagellate velocities (based on centers of mass for breakthrough curves).
[d] Collision efficiency factor describing interactions between flagellates and grain surfaces.
[e] ND, cannot be determined because breakthrough curves are incomplete for the most distal MLS.

also retarded and subject to enhanced dispersion relative to bromide (data not shown).

Comparison of dimensionless concentration histories for HE-labeled flagellate and bromide at M4A-15 (1.0 m down gradient from the point of injection) are depicted in Fig. 2A and B for the 9.0- and 9.3-m depths, respectively. For the 9.3-m depth, flagellates appeared 1 m down gradient within hours of the injection, and the initial peak in flagellate abundance preceded that of bromide by 1 day. However, when calculated on the basis of centers of mass for the entire breakthrough curve (Table 2), the flagellates appearing at M4A-15 within the time course of the experiment traveled at an average velocity that was only about one-fifth that of bromide. The flagellates were also subject to an approximately twofold-greater apparent dispersion than bromide. Approximately 13% of the flagellates that were injected into the aquifer appeared to have been transported 1 m down gradient within 45 days. This corresponded to a depth-averaged collision efficiency factor (α value) of 5.4×10^{-2}. Maximum flagellate concentrations at 1.0 m down gradient were only 2.1 and 1.4% of the injectate concentration, in contrast to maximum bromide concentrations of 67 and 99% at 9.0 and 9.3 m below land surface, respectively.

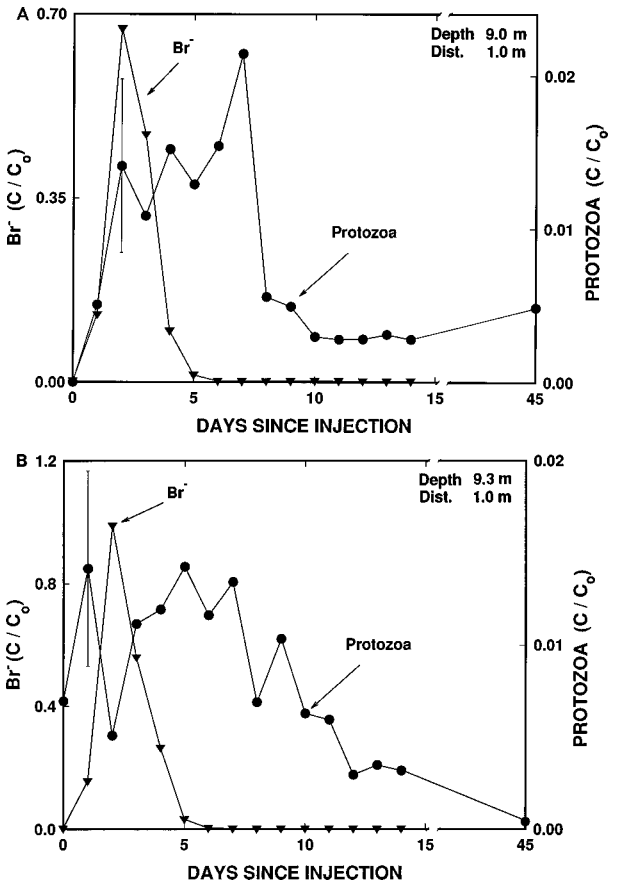

FIG. 2. Dimensionless concentration histories for HE-labeled flagellates and bromide in sandy aquifer sediments, 1 m down gradient from the point of injection, in a natural-gradient transport experiment at USGS MLS M4-15. Breakthrough curves are for the 9.0 (A)- and 9.3 (B)-m depths (below land surface). The error bar at first flagellate peak represents 1 standard deviation for replicate counts.

Concentration histories for stained flagellates and bromide at M5A-15 are shown in Fig. 3A and B for the 9.0- and 9.3-m depths, respectively. Substantial flagellate retardation, dispersion, and immobilization were still evident at 2.8 m down gradient from the point of injection, although they were considerably less than what was observed at 1 m down gradient. Flagellates appearing at M5A-15 had an average retardation and apparent dispersion of 2.4 and 11.3, respectively, which were only about half of what was calculated for the first meter of travel. Corresponding average bromide dispersion was 5.3. Although only 3% of the stained flagellates appeared to have been successfully transported 2.8 m down gradient from the point of injection, the loss of biomass that occurred over the intermediate 1.8-m distance between M4A-15 and M5A-15 was only 10% of what was injected, compared with an 87% loss over the first meter of travel. The average collision efficiency factor over the first 2.8 m of travel (3.4×10^{-2}) was less than two-thirds of what it was over the first meter. Differences in average groundwater velocity between the 9.0- and 9.3-m depths (0.47 versus 0.70 m day^{-1}) suggest the presence of heterogeneity in aquifer structure.

Breakthrough of stained flagellates and bromide at 3.6 m down gradient (M6-15) is depicted in Fig. 4A and B for the shallower and deeper depths, respectively. Although 98 to 99% of the biomass was immobilized over the entire 3.6-m transect, <2% was lost over the final 0.8-m travel distance between M5A-15 and M6-15. Dispersion factors for the flagellates could not be calculated, since a critical part of the breakthrough curve at 3.6 m down gradient from the injection point

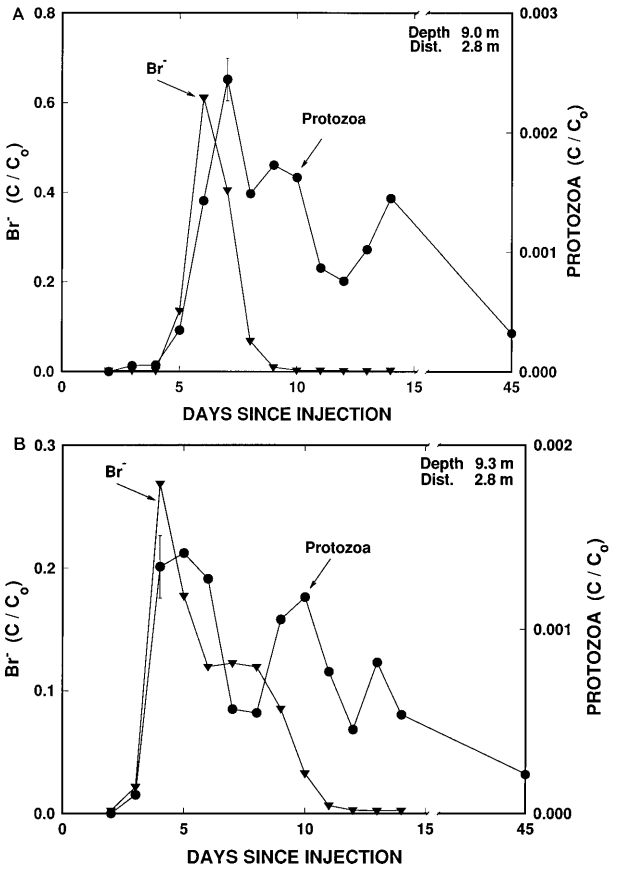

FIG. 3. Dimensionless concentration histories for HE-labeled flagellates and bromide in sandy aquifer sediments, 2.8 m downgradient from the point of injection, in a natural-gradient transport experiment at USGS MLS M4-15. Breakthrough curves are for the 9.0 (A)- and 9.3 (B)-m depths (below land surface).

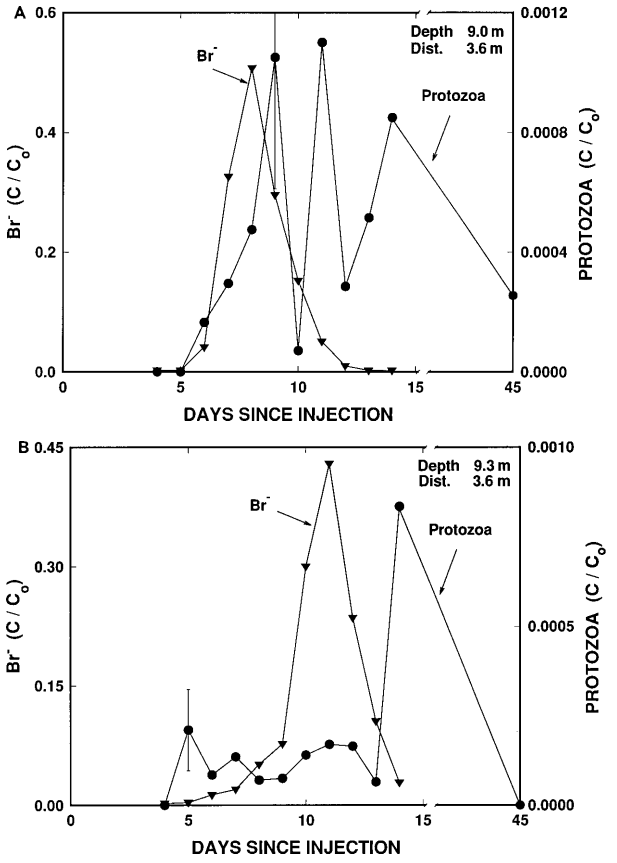

FIG. 4. Dimensionless concentration histories for HE-labeled flagellates and bromide in sandy aquifer sediments, 3.6 m downgradient from the point of injection, in a natural-gradient transport experiment at USGS MLS M4-15. Breakthrough curves are for the 9.0 (A)- and 9.3 (B)-m depths (below land surface).

was poorly defined. However, calculated longitudinal dispersions for bromide were 2.7 and 2.1 cm for the shallower and deeper depths, respectively.

Laboratory. Breakthrough curves for bromide and HE-stained flagellates are shown in Fig. 5 for a continuously injected, flowthrough column filled with sieved aquifer sediments. Detectable levels of bromide appeared after 0.8 pore volume had been collected (~2.5 h into the experiment). By 3 h (1.2 pore volumes), bromide broke through at the injectate concentration (i.e., $C/C_0 \cong 1.0$). In contrast, protozoa were not detected in column effluent until almost 6 h (1.8 pore volumes) into the experiment and did not reach peak abundance until 3.5 pore volumes had been collected. The maximum dimensionless concentration of protozoa was only ~0.1% that of bromide. Protozoan abundance in the column effluent never achieved steady state and fluctuated significantly over the course of the breakthrough. Because of the nonideal nature of the protozoan breakthrough curve, it was not possible to determine values of longitudinal dispersion or retardation from available models. However, judging from the late appearance of the protozoan peak, a retardation factor of ~3 is reasonable. Subsequent dissection of the column revealed that the immobilized HE-labeled flagellates were concentrated in the first 3 cm of the column sediments.

The effluent concentration history of each size class of microspheres for a similar flowthrough column experiment is depicted in Fig. 6. The intermediate-sized (1.7-μm) microspheres exhibited the highest dimensionless concentration in the effluent. At 26 h after initiation of injection, the dimensionless concentration of the smallest (0.7-μm) microspheres was 0.42, compared with 0.83 for the 1.7-μm microspheres.

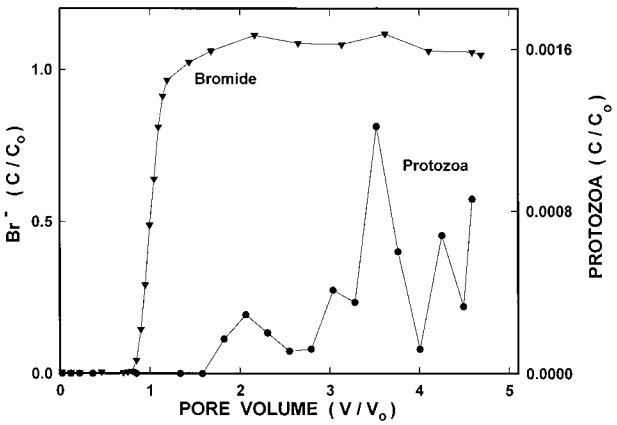

FIG. 5. Dimensionless concentration histories for HE-labeled flagellates and bromide in the effluent of a flowthrough column packed with sieved (0.5- to 1.0-mm grain size) aquifer sediments. The column was run in the upflow mode at ~2 ml · min^{-1}, 10°C, and 10° to the horizontal.

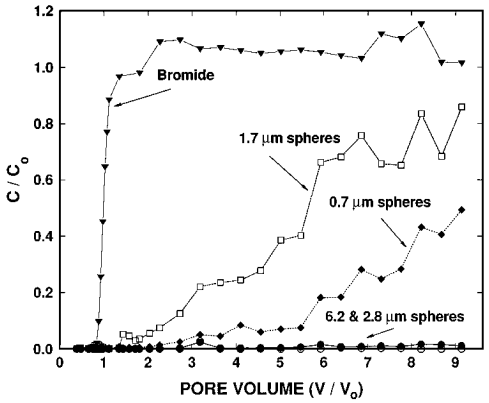

FIG. 6. Dimensionless concentration histories for 0.7-, 1.7-, 2.8 (●)-, and 6.2 (○)-μm (diameter) carboxylated microspheres and bromide in the effluent of a flowthrough column packed with sieved (0.5- to 1.0-mm grain size) aquifer sediments. The column was run in the upflow mode at ~2 ml · min^{-1}, 10°C, and 10° to the horizontal.

The largest microspheres (2.8 and 6.2 μm) were not significantly transported; none of the 6.2-μm and few of the 2.8-μm spheres were successfully advected through the 0.6 m of sediment to the effluent end of the column.

DISCUSSION

Relative transport behavior. Although larger than the 2- to 3-μm flagellates found in the Cape Cod aquifer, encysted *Giardia* (8 by 12 μm) and *Cryptosporidium* (4 μm) organisms are occasionally detected in well water samples (34), suggesting groundwater transport of introduced protozoa under special circumstances. However, little information about the transport behavior of protozoa is available in the subsurface microbial transport literature, which focuses largely upon bacteria and viruses (4, 42). Because of the dearth of information on protozoan transport behavior in aquifer sediments and because of the potential importance of protozoan-bacterial interactions in the ecology of the Cape Cod aquifer, it is useful to compare transport behavior of HE-stained flagellates in this experiment with that observed for 4′,6-diamidino-2-phenylindole (DAPI)-stained bacteria in earlier small-scale (1- to 7-m [distance]) studies.

It is evident from the retardation (Table 2) and broad spreading of the flagellate breakthrough curves (relative to those of the conservative tracer) that most of the flagellates observed at 1.0, 2.8, and 3.6 m down gradient from the point of injection (Fig. 2, 3, and 4) seemed to have interacted with grain surfaces along the path of travel. In contrast to the disparity between bromide and flagellate concentration histories, breakthrough curves for indigenous bacteria appearing down gradient in nearby forced- and natural-gradient experiments (12, 13) were similar in pattern and peak arrival time to those of the conservative tracer (bromide or chloride). In the Harvey and Garabedian study (12), the peak arrival of stained bacteria and that of bromide appeared to be coincident, although the tailing in the bacterial breakthrough curve was slightly more protracted than that observed for bromide. Near-coincident arrival of peak microorganism abundance with bromide was also observed in a recent, nearby injection and recovery experiment involving bacteriophage (PRD1) transport (1). In the latter experiment, the apparent dispersion of the virus was close to that observed for the conservative tracer. Therefore, labeled flagellates transported downgradient from the injection well in the test described here were subject to a greater number of interactions with grain surfaces per meter of travel and/or their average residence time on grain surfaces was greater than that experienced by the bacteria or the virus.

The importance of grain-surface interactions in the subsurface transport behavior of flagellates is also supported by the high rate of flagellate immobilization in aquifer sediments observed in both field (Table 2) and flowthrough column (Fig. 5) experiments. In the field, there was an order of magnitude loss in free-swimming flagellates over the first meter of travel compared with an order of magnitude loss for the first 10 m of bacterial transport in an earlier experiment (12). However, in the absence of motility, indigenous bacteria would predictably contact grain surfaces with substantially greater frequency than would groundwater flagellates (assuming both were near neutral buoyancy). This is because the bacteria (0.6 μm, average cell length [13]) would be subject to greater Brownian movement on the basis of their smaller size. However, the lower collision efficiency factors for bacteria (5.4×10^{-3} to 8.5×10^{-3} [12]) relative to those observed for flagellates (2.6×10^{-2} to 6.9×10^{-2}; Table 2) suggest that flagellate collisions with grain surfaces are more likely to result in attachment. Flagellates with high buoyant density (e.g., 1.10 g · cm^{-3}) would contact grain surfaces with greater frequency because of a stronger influence of settling, but probably not enough to account for the large differences in immobilization rate between bacteria and flagellates in small-scale tests. The buoyant density of the flagellates is unknown. However, they appear to be subject to rates of sinking in the aquifer similar to the 2-μm, 1.05-g · cm^{-3} carboxylated microspheres (14).

Although microsphere transport behavior in aquifer sediments differed markedly from that of indigenous groundwater bacteria (13), there were a number of similarities between the transport characteristics of carboxylated microspheres and groundwater flagellates. Both the microspheres (Fig. 1) and the flagellates (Fig. 2, 3, and 4) seem to be subject to substantial retardation, immobilization, and enhanced dispersion relative to a conservative tracer, suggesting a high propensity for reversible and irreversible interactions with grain surfaces. Enhanced apparent dispersion, retardation, and immobilization relative to bromide were also observed for other size classes of carboxylated microspheres in field transport experiments (13, 16). Both microspheres and flagellates exhibited irregular, multipeaked concentration histories (Fig. 1 to 4) in comparison to those observed for bromide. The collision efficiency factor calculated for carboxylated microspheres (2.6×10^{-2}) (12) in a nearby natural-gradient transport experiment was within the range of that calculated for the flagellates in this study (2.6×10^{-2} to 6.9×10^{-2}; Table 2).

The similarities in transport behavior between flagellates and carboxylated microspheres suggest that the latter may be used in laboratory and field studies assessing abiotic controls of flagellate motility. The difficulties in constructing a model that would accurately describe the retarded, discontinuous manner in which groundwater flagellates apparently move through the aquifer enhance the attraction of using carboxylated microspheres as analogs, particularly for in situ injection and recovery studies. The advantage of using brightly fluorescing microspheres is that they are easy to detect by fluorescence microscopy, chemically stable, and easy to handle in the field and in the laboratory. The comparatively dull fluorescence and fragile nature of the HE-stained flagellates may render their use in field transport experiments more difficult, particularly in longer experiments. The disadvantage of using microspheres is that they cannot provide information on how flagellate mobility in the aquifer is affected by environmental stimuli. The

potential use of microspheres as transport analogs for nonmotile, highly resistant cysts of groundwater flagellates may be particularly promising and will be assessed in future field transport experiments. Microspheres may also be useful in assessing the transport behavior of oocysts of the protozoan pathogen, *Cryptosporidium parvum*.

Nature of immobilization. Physical straining (entrapment within intergranular spaces smaller than the limiting dimension of the organism) does not appear to be much of an impediment to flagellate mobility in Cape Cod aquifer sediments on the basis of the relative sizes of the flagellates compared with the diameters of the sand grains with which they are interacting. Straining in homogeneous porous media would be expected to occur for the largest of our cultured flagellates (5 μm) if the grain diameter was on the order of 100 μm or smaller, assuming that the colloidal diameter must be at least 5% of that of the collector, as noted by McDowell-Boyer et al. (30). However, mean grain size in the aquifer is much larger, i.e., ~0.59 mm (25). A more rigorous approach may be used to predict microbial straining in nonuniform sediments (29). The latter approach compares the microbial cell's size with the critical pore size (calculated on the basis of the grain size frequency distribution and porosity) and assumes that straining occurs when this ratio is 1.5 or higher. Assuming a grain size distribution comparable to those reported by Barber (2) for the area of the aquifer in which our small-scale test was conducted, the ratio of flagellate to critical pore size would be substantially less than 1.

Straining of microorganisms moving through heterogeneous, sandy sediments is still a possibility, if the path of transport intercepts pockets of finer material (silts and clays). Although relatively devoid of clay, the aquifer at the Cape Cod site is characterized by lenses of fine sand interspersed in layers of coarse sand and gravel (26). However, even with removal of fine sands (<0.5-mm grain diameters) from bulk aquifer sediments, flagellate immobilization in the laboratory was as great or greater than that observed in the field (Fig. 5). Therefore, it does not appear that straining played an important role in the small-scale transport experiments.

It is likely that the high rate of flagellate immobilization in these sediments is greatly affected by the organisms' propensity for solid surfaces. Flagellate adherence to solid surfaces is species specific, and some soil flagellates prefer the free-swimming state (34a). However, flagellates in the Cape Cod aquifer are almost all surface associated (23), even in areas of the contaminant plume where a substantial portion of the bacterial population is unattached (11). It has been our experience that flagellates collected from the aquifer require special caution in handling during the culturing and staining procedure in order to preclude attachment to each other via flagellar entanglement, to glass, and to tubing (22). The propensity of the flagellates for grain surfaces in the aquifer sediments may be due, in part, to the physicochemical nature of their two flagella and to the surface-associated manner in which they are thought to feed in natural aquatic habitats (7). The fact that detectable numbers of stained flagellates were observed 3.6 m down gradient 1 month after the conservative tracer could no longer be detected suggests that many flagellate-grain interactions are reversible in nature.

Temporal and physical variability. Physical variability in aquifer structure has been shown to affect the relative transport behavior of bacteria and microspheres in a recent injection and recovery experiment at the Cape Cod site (16) and of bacteria in flowthrough columns (8). In the former study, the bacterial peak at 6 m down gradient from the point of injection was observed to precede, lag, or be coincident with the peak in bromide, depending on the sampling depth and, consequently, on the characteristics of the layer of sediments through which the injectate moved. Considerable information has been collected on the effects of aquifer heterogeneity on the large-scale transport of conservative tracers in the Cape Cod aquifer (10, 18, 26). However, because it is not practical to study microbial transport in the Cape Cod aquifer on such a large scale (over a 100-m transport distance), it is important in small-scale studies to assess relative microbial transport behavior at a number of points downgradient from the injection well. This allows the reasonable establishment of trends in transport behavior that are not mere artifacts of the physical heterogeneity of the system.

The early appearance of the initial peak in flagellates relative to that of bromide at the 9.3-m depth of M4A-15 (Fig. 2B) compared with the later flagellate peaks at the other sample points suggests the influence of physical variability in aquifer structure. The effects of physical variability on the transport of the conservative tracer also may be seen in Fig. 3B, in which there is a substantial shoulder on the breakthrough curve for bromide at the 9.3-m depth of M5A-15. The appearance of large numbers of labeled flagellates shortly after injection and before any bromide was detected (Fig. 2B) suggests that the pore volume available to the flagellates was measurably smaller than that available to the conservative tracer. This can be caused by internal porosity within the grains themselves into which the bromide, but not the protozoa, can penetrate or by preferred flow path structure. In spite of the physical heterogeneity, it is evident that in all cases the centers of mass of the labeled flagellates being advected down gradient were substantially retarded relative to that of bromide. Also, at all of the sampled points, the flagellates were subject to substantially higher degrees of apparent dispersion relative to bromide.

Table 2 suggests that flagellate transport characteristics may change over the experimental time course. In general, the flagellates appear to be more mobile (less surface active) with increasing distance of travel. Much of the change in cell size and rate of immobilization appears to take place within the first meter of travel. Therefore, flagellate transport over the more distal segments of the transect (1.0 to 3.6 m down gradient from the point of injection) may be more indicative of the true manner in which these organisms move through the aquifer. Temporal changes in cell characteristics during the month-long experiment cannot be overlooked in the interpretation of the results, because the flagellates were transferred from culture media to carbon-limited (39) conditions in the aquifer. It appears that physiological changes may have taken place, particularly during the first week of the experiment. Although the laboratory-grown flagellates had a size distribution of between 4 and 5 μm, the HE-stained flagellates appearing down gradient were all ~2 μm. In flowthrough column experiments involving different size classes of microspheres (Fig. 6), the 3- to 6-μm size classes appeared to be relatively immobile, whereas the 2-μm spheres appeared in the column effluent in concentrations that approached 85% that of the injectate. Preferential transport of the 1.7-μm spheres is consistent with the colloid filtration theory (33) and with one of two earlier flowthrough column experiments involving a polydispersed mixture of carboxylated microspheres in the 0.5- to 5-μm size range (16).

Although the surface characteristics of the unadapted, laboratory-grown flagellates are likely different from those of comparably sized microspheres, it can be predicted from physical considerations that the largest flagellates would be at a disadvantage for advective transport because of greater settling (12). The size range of the more mobile HE-labeled flagellates in the field test is similar to the flagellate size range observed

in protozoan surveys of the Cape Cod aquifer, i.e., 2 to 3 μm (23). We believe that the marked decrease in flagellate cell size with travel down gradient reflects physiological changes in response to changes in the nutritional and/or physical environment, because the size class found down gradient (2 μm) was not present in the injectate. Also, the flagellates remaining around the point of injection (M4-15) underwent a similar decrease in cell size over the time frame of the experiment, in spite of their apparent lack of down gradient travel. More recent laboratory studies indicate that flagellate cell size is highly dependent on its physical and nutritional environment. Higher-nutrient liquid medium selects for 4- to 5-μm flagellates, whereas growth in lower-nutrient porous medium (sieved aquifer sediments) tends to select for smaller, in situ (2- to 3-μm) cell size (22).

In summary, it appears that the large population of flagellates that inhabits the aquifer sediments at Cape Cod is capable of movement in the direction of flow. Movement by advection as described here is orders of magnitude greater than that possible by diffusion alone. However, the flagellates we tested move in a manner that is substantially more retarded, dispersed, and attenuated than that of the free-living indigenous bacteria. The flagellates' high propensity for grain surfaces in the aquifer and in flowthrough column experiments is consistent with our observations that few flagellates in the aquifer are free-swimming, even in the contaminated zones where their overall abundance in aquifer sediments is quite high (10^5 of dry weight^{-1}). Although not tested, transport potential in the Cape Cod aquifer for protozoan pathogens, which tend to be much larger than our groundwater flagellates, would appear to be low. In the small-scale field experiment, temporal changes in flagellate size and mobility suggest that some readaptation to aquifer conditions can occur over relatively small distances (e.g., within 1 m). Although it is not possible to collect enough flagellates directly from well water to run transport experiments in the field, a new growth technique involving culturing in lower-nutrient, porous media (22) will allow use of in situ-sized groundwater flagellates in future groundwater experiments. The transport behavior of flagellate cysts and the role of motility in flagellate dispersal within aquifer sediments are not known and will be explored in future small-scale injection and recovery studies.

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