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Physiological Considerations in Applying Laboratory-Determined Buoyant Densities to Predictions of Bacterial and Protozoan Transport in Groundwater: Results of In-Situ and Laboratory Tests

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Buoyant densities were determined for groundwater bacteria and microflagellates (protozoa) from a sandy aquifer (Cape Cod, MA) using two methods: (1) density-gradient centrifugation (DGC) and (2) Stoke's law approximations using sedimentation rates observed during natural-gradient injection and recovery tests. The dwarf (average cell size, 0.3 μm), unattached bacteria inhabiting a pristine zone just beneath the water table and a majority (~80%) of the morphologically diverse community of free-living bacteria inhabiting a 5-km-long plume of organically-contaminated groundwater had DGC-determined buoyant densities <1.019 g/cm^3 before culturing. In the aquifer, sinking rates for the uncultured 2-μm size class of contaminant plume bacteria were comparable to that of the bromide tracer (1.9×10^{-3} M), also suggesting a low buoyant density. Culturing groundwater bacteria resulted in larger (0.8−1.3 μm), less neutrally-buoyant (1.043−1.081 g/cm^3) cells with potential sedimentation rates up to 64-fold higher than those predicted for the uncultured populations. Although sedimentation generally could be neglected in predicting subsurface transport for the community of free-living groundwater bacteria, it appeared to be important for the cultured isolates, at least until they readapt to aquifer conditions. Culturing-induced alterations in size of the contaminant-plume microflagellates (2−3 μm) were ameliorated by using a lower nutrient, acidic (pH 5) porous growth medium. Buoyant densities of the cultured microflagellates were low, i.e., 1.024−1.034 g/cm^3 (using the DGC assay) and 1.017−1.039 g/cm^3 (estimated from in-situ sedimentation rates), suggesting good potential for subsurface transport under favorable conditions.

An important property governing microbial mobility in groundwater environments is buoyant density (specific gravity). A microorganism's buoyant density affects both its frequency of collision with grain surfaces during advective transport and its downward movement in response to gravity (rate of settling). Depending upon the size of the microorganism, buoyant density can be an important determinant of its rate of immobilization, retardation, and sedimentation to adjacent conductive layers within sandy aquifer sediments. However, there is much uncertainty in choosing representative buoyant densities for transport models used to predict movement of microorganisms through saturated porous media. For *Pseudomonas aeruginosa* being advected through saturated porous media, buoyant densities of 1.04, 1.10, and 1.13 g/cm^3 were applied to predictions of deposition (*1*). These values of buoyant density were chosen because most laboratory measurements performed on cultured bacterial populations tend to fall within this range (*2*). However, in other modeling studies, neutral or near-neutral buoyancy is assumed for the bacteria being transported through aquifer sediments and (or) soils (*3*−*5*). Many of the bacterial buoyant density values reported in the literature derive from laboratory studies performed on large, cultured isolates of clinical interest (e.g., refs 6 and 7). The use of more environmentally-relevant values of buoyant density in subsurface transport models has been hampered by a dearth of information on the properties of indigenous groundwater bacteria.

Considerable progress toward a data base for buoyant densities of groundwater bacteria was made in a recent study in which buoyant densities were carefully determined for a variety of cultured, groundwater bacteria (*8*). Twenty-five isolates, collected largely from the Middendorf aquifer (South Carolina) and from shallow groundwater at Oyster, VA (courtesy of Dr. A. Mills, University of Virginia), were used. The buoyant densities of the cultured isolates ranged from 1.04 to 1.12 g/cm^3, with an average value of 1.09 g/cm^3. The range of buoyant densities reported in the above study seems to be consistent with observations using cultured bacteria from other environments and similar experimental procedures (*2*). Although buoyant densities substantially greater than 1.00 g/cm^3 may be necessary for modeling initial subsurface transport of cultured groundwater bacteria, it is unclear whether such values should be used to predict subsurface transport behavior of uncultured groundwater bacteria. It is also unclear whether such values are applicable to groundwater bacteria grown in the lab, re-introduced into the aquifer, and allowed sufficient time to re-adapt to in-situ conditions. Observations that labeled, uncultured groundwater bacteria can behave in aquifer sediments as though they are near-neutral buoyancy (*9*) suggest the possibility that the buoyant densities of uncultured groundwater bacteria may differ significantly from those of cultured isolates. The alterations in physical characteristics such as buoyant density, cell size, and surface properties resulting from growth in liquid broth (LB) are not well understood for groundwater microorganisms, but have important ramifications for subsurface bacterial transport and, consequently, for aquifer bioresto-ration.

Our first objective was to determine whether there are substantive differences between buoyant densities of cultured and uncultured groundwater bacteria. This was accomplished by comparing buoyant densities reported by Wan et al. (*8*) for a number of bacteria isolated from aquifers in the southeastern United States and grown in LB to those observed for uncultured, free-living bacteria inhabiting organically-contaminated and uncontaminated zones of another sandy aquifer (Cape Cod, MA). Buoyant densities for the uncultured groundwater bacteria were determined using two methods: (1) the density-gradient centrifugation (DGC) technique employed by Wan et al. (*8*) and (2) the observed sedimentation rates (relative to a conservative tracer) in a small-scale, natural-gradient injection and recovery experiment (October 1987). We also used the DGC technique to compare buoyant

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densities of cultured isolates with those of uncultured bacterial populations from which the isolates originated.

The second objective was to determine the buoyant densities for the 2−3-μm microflagellates (protozoa) that appear to be an important component of the protistan community in the Cape Cod aquifer (*10*). Little information is available on the physical properties of these ecologically important protists. The microflagellates are near optimal size for transport in Cape Cod aquifer sediments, judging from flow-through column experiments with different (0.7−6.2 μm) microspheres (*11*) and predictions based upon colloid-filtration theory using values of 0.59 mm for median grain size, 0.33 m/d for groundwater velocity, and 288 K for groundwater temperature (*2*, *4*). However, transport behavior in this size range can be significantly affected by buoyant density, which controls their rate of settling during advective and tactic movement. Microflagellate buoyant densities, determined by using the aforementioned DGC technique, were compared to observed sedimentation rates in the 1994 small-scale injection and recovery field test.

Experimental Section

Collection of Indigenous Bacteria and Protozoa. The site of this study is located on Cape Cod, MA, where there is an unconfined, sandy aquifer. Mean grain size, average porosity, and hydraulic conductivity are ~0.5 mm, 0.38, and ~0.1 cm/s, respectively (*12*). A 5-km-long plume of organic contaminants has resulted from the surface discharge of secondary effluent from a treatment plant on the boundary of Otis Air Base. The contaminant plume is the site of a multidisciplinary research program administered by the U.S. Geological Survey (USGS) and is characterized by elevated levels of specific conductance (up to 400 μS cm^{-1}), dissolved organic carbon (DOC) (up to 4 mg mL^{-1}), and temperature (up to 18 °C) relative to adjacent uncontaminated groundwater (<80 μS cm^{-1}, <1 mg/L, and 10 °C, respectively). It serves as the habitat for a large ($\leq 10^7$ mL^{-1}) and morphologically diverse community of free-living bacteria. Unattached bacteria for the respective field injection and DGC assays were concentrated directly from groundwater by tangential flow (*13*) or by direct filtration (*14*). Groundwater from contaminated and uncontaminated zones of the aquifer was collected in sterile containers from 5-cm (diameter) PVC observation wells using a Keck submersible pump and Teflon tubing. Contaminated groundwater was collected from the contaminant plume at USGS well F314-51, which samples the contaminant plume 100 m downgradient from point of discharge, and from USGS well F350-13, which samples uncontaminated groundwater (above the plume, 3 km downgradient from well F314-51). Groundwater samples from which bacteria were concentrated for the DGC buoyant-density assays were shipped on ice by overnight courier to the USGS laboratories in Boulder, CO.

Because of the low numbers of unattached microflagellates recovered in well water (*15*), microflagellates for the buoyant-density determinations were harvested from cultures. Core material containing a diverse population of microflagellates from the contaminated zone 2−3 m below the water table at USGS well site F230 was recovered aseptically and in the absence of drilling fluids using a wireline piston-type coring device in conjunction with a hollow-stem auger drill (*16*). An aquifer sediment inoculum was then subsampled from the intact core. A culturing procedure (*17*) was employed to grow enough microflagellates to do an injection and recovery experiment and to do a DGC assay for buoyant density. The method involved growth in porous media (sieved aquifer sediments) under low nutrient and in-situ pH conditions and resulted in microflagellates that were similar in size to those observed in-situ (2−3 μm). The cultures also contained bacteria that were in the initial inoculum and grew in the medium. In contrast, the higher nutrient, liquid culturing procedure used to grow a flagellate population for an earlier field-transport experiment (*11*) typically resulted in cells that were substantively larger (i.e., 4−5 μm).

Average Size Determinations. Size frequency analyses of free-living bacteria were done using a Nikon Optiphot II (Nikon, Buffalo, NY) epifluorescence microscope and an ITC (Image Technology Corporation, Deer Park, NY) image processor connected to a personal computer, a Dage SIT66 black and white camera, and a Sony black and white monitor. The image system was optimized to analyze and calculate length, width, area, and perimeter of fluorescently (Acridine Orange) stained bacteria in samples previously analyzed for bacterial abundances. Measurements from the image system were standardized using fluorescently stained 0.95, 1.07, and 0.45 μm microspheres (Polysciences) in order to convert pixel measurements to micrometers. All analyses were performed at microscope magnifications of 788−1260×.

Buoyant-Density Estimates. *Density-Gradient Centrifugation*. Prior to the buoyant-density determination, bacteria or microflagellates were concentrated, resuspended in sterile saline media, added to pre-formed gradients, and centrifuged. For bacteria, approximately 6 L of uncontaminated groundwater or 1 L of contaminated groundwater was filtered through 47-mm (diameter), 0.2-μm (pore size) polycarbonate membrane filters (Nuclepore Corp.) at ~0.3 atm (transmembrane pressure). Bacteria collected on the filters were gently washed with sterile saline solution (2 mM NaCl, pH 6.8) and resuspended in sterile 0.15 N NaCl to give a cloudy suspension consisting of $\sim 1 \times 10^9$ cells/mL. Microscopic examination revealed that the vast majority of bacteria were single and not attached to particles. Few non-bacterial colloids were observed. The microflagellates were concentrated from the interstitial fluid of the amended porous medium using filtration (0.8 μm) under low transmembrane pressure (< 0.3 atm) and resuspended using the same technique. Aquifer bacteria ≥0.8 μm that proliferated in the interstitial fluids of the flagellate, porous-media culture were also present in the final flagellate resuspensions.

Buoyant-density determinations were performed using the method of Wolff (*18*). Density gradients were created within transparent, 50-mL Oak Ridge or 10-mL polycarbonate centrifuge tubes (Nalgene) using Percoll I solution (1.131 g/mL, Sigma Chemical Company), a colloidal silica suspension, diluted with 0.15 M NaCl. The tubes were then spun for 30 min at 15000*g* in a Sorvall RC-5B refrigerated centrifuge (Dupont). The resulting gradient formed symmetrically on either side of the starting density (1.100 g/mL). Brightly colored density marker beads (Sigma Chemical Company) were used to indicate specific buoyant-density values along the longitudinal axis of the tubes. Measured (2.5 mL) aliquots of the bacterial or flagellate suspensions were carefully layered on the top of the pre-formed gradients. Gradient tubes containing the bacteria were spun at 15000*g* for 1 h. Those containing the microflagellates, which are more likely to lyse when subjected to high centrifugal force, were spun at 8000*g* for 2 h. The equilibrium positions of the bacterial or flagellate populations were observed as distinct translucent bands. The buoyant densities of the populations were indicated by the position of the bacterial or microflagellate bands relative to those of the marker beads.

To determine the effect of culturing in liquid media on the buoyant densities of groundwater bacteria, samples of the visibly-cloudy bacterial bands were carefully withdrawn by syringe from the transparent centrifuge tubes containing the Percoll gradient and marker beads. Groundwater bacteria were then isolated by plating out samples from the Percoll gradient on dilute soil-extract agar (DSEA), which consisted of 10% soil extract broth, 2% glucose, and 3% agar suspended in filter-sterilized groundwater. Selected colonies appearing on the agar plates were removed, and the bacteria therein were subsequently grown in liquid media consisting of dilute

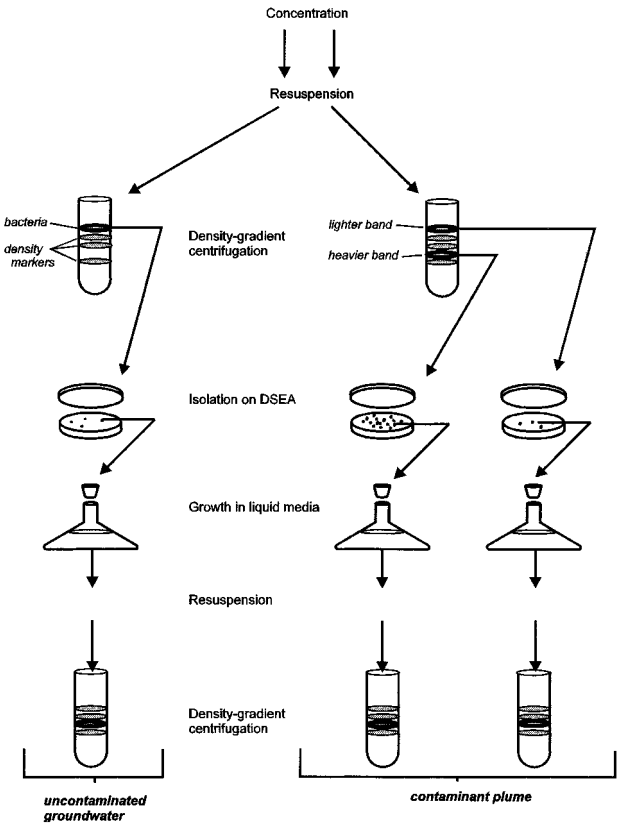

FIGURE 1. Protocol used to determine the buoyant densities of indigenous groundwater bacteria collected from contaminated and uncontaminated zones of a sandy aquifer in Cape Cod, MA. DSEA is diluted soil extract agar.

soil-extract media (10% soil extract broth and 2% glucose) suspended in filtered (0.2 μm) groundwater. To make the soil extract broth, 100 g of uncontaminated aquifer sediments was added to 100 mL of deionized water, autoclaved for 15 min at 120 °C, filtered (0.2 μm pores size), and diluted 1:10 with filter-sterilized groundwater. It has been shown that the buoyant density of a cultured isolate can be significantly affected by high concentrations of inorganic salts in the growth medium (*6*, *7*). For example, it was reported that increasing the NaCl concentrations in the suspending medium from 0.009 M to 0.9 M resulted in an increase in buoyant density of *Escherichia coli* B/r NC32 from 1.086 to 1.105. To lessen this effect, we did not add inorganic buffers to the growth medium. The buoyant density for the cultured bacterial isolates was determined by resuspension in dilute saline (non-growth) solutions, addition of the suspension to pre-formed gradients, and centrifugation at 15000*g* as described above. The protocol for the buoyant-density determination of uncultured and cultured groundwater bacteria is depicted in Figure 1.

In-Situ Sedimentation Rates. Buoyant densities for the uncultured bacteria and the cultured flagellate populations were also estimated from their rates of sedimentation in the aquifer relative to a conservative tracer. Unattached bacteria were collected from the contaminant plume and concentrated at the well head using a microtubule-type tangential-flow filtration device described by Kuwabara and Harvey (*13*). The subsequent bacterial suspension was then labeled with 5 μM (final concentration) 4,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Company), a DNA-specific fluorescent dye. The 2-L suspension of labeled, indigenous bacteria was then added to 100 L of groundwater collected from the injection depths (8.5 and 9.1 m below land surface). The bacterial suspension was then amended with bromide, which served as a conservative tracer to account for changes in altitude along the travel path due to the hydrologic gradient and to physical heterogeneities. The bromide was added in trace quantities (1.9×10^{-3} M, maximum concentration before dispersion in the aquifer) so as not to substantively affect the specific gravity of the groundwater. The injectate was added to the aquifer under natural-gradient conditions, and the bromide and bacterial concentrations were monitored at various depths at 6.7 m downgradient by temporally sampling appropriate ports of multilevel samplers (MLS) that were along the trajectory of the injectate cloud. The rate of bacterial sedimentation was determined by comparing the altitude of the center of mass for the unattached, labeled bacteria to that of bromide at the downgradient MLS.

Aquifer flagellates were cultured using the aforementioned low-nutrient, porous-media growth technique and harvested from interstitial fluids withdrawn from the porous media, fixed with formaldehyde (1% v/v final concentration), and stained with DAPI. The stained microflagellates were added to 100 L of groundwater collected from the injection depths. The injectate was also amended with bromide (1.9×10^{-3} M, final concentration) before being injected as a pulse to the aquifer at USGS well M4-15. The injection and recovery test was performed concomitantly with a virus transport experiment described by Pieper et al. (*19*). For the purposes of determining sedimentation rates, labeled microflagellates and bromide that were injected at an altitude of 10.97 m above sea level were monitored as they moved within a three-dimensional grid of MLS sampling ports, spaced ~25 cm apart in the vertical direction and ~ 1 m apart in the direction of flow. Comparisons were made between the sedimentation rates for bromide and for the unretarded labeled microflagellates. This was done by determining the altitudes and times of appearance of the respective centers of mass at MLS M6-15, located 3.6 m directly downgradient from the injection well.

The buoyant densities of the bacteria and microflagellates were estimated from the relationship proposed by Wan et al. (*8*) to account for the influence of grains upon sedimentation velocities of colloidal-sized particles in a porous medium, i.e.

$$v \approx \left(\frac{b + 0.67}{b + (0.93/\epsilon)}\right) v_0 \qquad (1)$$

where v is the average sedimentation velocity in porous medium, b is the ratio of average free sedimentation segment length to grain radius, ϵ is an empirical correction factor arising from influences of the grain surface, and v_0 represents the maximum possible instantaneous sedimentation velocity. As noted by Wan et al. (*8*), the presence of rounded grains do not have a major effect upon sedimentation velocity when grains contribute only to tortuosity. For an assumed $b \geq 1$ and $\epsilon = 1$, v should be within 86−100% of v_0. The latter value represents the theoretical sedimentation velocity experienced by a perfect sphere in a column of water and is given by the well-known steady-state solution of Stoke's equation:

$$v_0 = \frac{(\rho_p - \rho) g d_p^{\,2}}{18\mu} \qquad (2)$$

where μ is the dynamic fluid viscosity, ρ_p and d_p are the respective buoyant density and diameter of the sedimentation particle (in this case, groundwater bacteria), ρ is the fluid density, and g is the acceleration due to gravity. For describing sedimentation rates of microorganisms, the Stoke's equation is sometimes modified by the addition of a Φ term in the denominator (*20*), which accounts for a microorganism's nonspherical shape. However, because the relationship between morphology and sedimentation velocity is not fully understood, this modification of the Stoke's equation is not used here.

TABLE 1. Buoyant Densities for Groundwater Bacteria and Flagellates As Determined by Density Gradient Centrifugation

location[a]	sample or inoculum	description	avg. size[b] (μm)	buoyant density (g/cm^3)[c] 1° band[d]	2° bands[e]
F350-13	pristine groundwater	uncultured free-living bacteria	0.3	<1.019	none
		cultured isolate 1 (from primary band)	1.3	1.043	
		cultured isolate 2 (from primary band)	0.8	1.081	
F314-51	contaminated groundwater	uncultured free-living bacteria	0.6	<1.019	1.076
		cultured isolate 3 (from primary band)	1.4	1.082	
		cultured isolate 4 (from secondary band)	1.0	1.088	
F230	aquifer sediments	cultured, mixed flagellate populations (porous-media grown)	2.5	1.024−1.034	1.040−1.075

[a] Refers to well at the U.S. Geological Survey Cape Cod groundwater hydrology study site. [b] As determined by digital-image analysis of fluorescently stained microbes on a black membrane filter. [c] Standard errors associated with density differences between bacteria and water are generally <10% (*8*). [d] Microbe-containing band containing the majority of the biomass that was added to the density-gradient tube. [e] Microbe-containing band containing a smaller segment of the biomass that was added to the density-gradient tube.

TABLE 2. Calculated Buoyant Densities for Groundwater Bacteria and Flagellates As Determined from Observed Sedimentation Rates in Small-Scale, Natural-Gradient Injection and Recovery Experiments

date	location[a]	organism	travel distance (m)	size (μm)	buoyant density (g/cm^3)[b]
October 1987	F347	morphologically-diverse populations of uncultured free-living bacteria concentrated from contaminated groundwater	6.7	0.2−1.6	<1.02[c]
June 1995	M4-15	mixed populations of groundwater flagellates cultured in porous-media under low pH conditions	3.6	2−3	1.017−1.039

[a] Refers to well at the U.S. Geological Survey Cape Cod groundwater hydrology study site. [b] Standard errors associated with density differences between bacteria and water are generally <10% (*8*). [c] Determined for the 2-μm size class. Buoyant-density determinations for the smaller bacteria could not be made from field data because the vertical spacing between sampling ports would not be close enough.

Results

Laboratory Determinations. Laboratory-determined buoyant densities for groundwater bacteria and microflagellates are listed in Table 1. Using the DGC technique, substantial differences between the buoyant densities of cultured and uncultured bacterial populations are apparent. The uncultured, free-living bacteria collected from the uncontaminated zone formed a band that corresponded to a buoyant density slightly less than that of our most neutrally-buoyant marker beads (1.019 g/cm^3). Similarly, most of the uncultured, morphologically-diverse free-living bacteria collected from within the contaminant plume were also quite "light" and formed a distinctive band within the density-gradient tube corresponding to buoyant density <1.019 g/cm^3, although a faint secondary band corresponding to a buoyant density of ~1.08 g/cm^3 was also observed. In contrast to the uncultured bacteria, the cultured isolates from the uncontaminated groundwater had buoyant densities of 1.043 and 1.081 g/cm^3. Cultured bacteria isolated from the free-living community inhabiting the contaminant plume had a buoyant density of 1.082 and 1.088 g/cm^3. Three of the four cultured bacteria were originally isolated from the near-neutral buoyant bacterial populations comprising the primary bands in the DGC tubes. The buoyant densities for the four cultured groundwater isolates (Table 1) are comparable to those of the eight other cultured isolates used in the process of methods optimization for maximizing density band separation (data not shown).

Epifluorescence microscopy revealed that a majority of the groundwater microflagellates, cultured in sieved aquifer sediments, were in a narrow buoyant-density band between 1.027 and 1.034 g/cm^3. However, a majority of the bacteria that also proliferated within the porous-media culture were located in a second, broad band corresponding to a range of buoyant densities of 1.040−1.075 g/cm^3. Few microflagellates were observed in the latter (secondary) band.

Average cell sizes, as determined using fluorescence microscopy and digital image analysis, are also listed in Table 1. The uncultured bacteria inhabiting the uncontaminated zone of the aquifer near the water table were quite small (average cell size 0.3 μm). However, the bacteria cultured from this zone were substantially larger (0.8 and 1.3 μm). Similarly, the cultured bacteria isolated from the contaminant plume (1.0 and 1.4 μm) were considerably larger than average size for the morphologically diverse bacterial community from which they were isolated (0.6 μm). The average size of the porous-media-grown microflagellates was ~2.5 μm, which is within the range of its observed in-situ size (2−3 μm).

Field Determinations. Bacterial and flagellate buoyant densities, as determined by observed rates of sedimentation in small-scale injection and recovery experiments, are listed in Table 2. The morphologically-diverse bacteria concentrated from the contaminant plume and labeled with DAPI appeared to be near-neutral buoyancy, judging from their negligible rate of sedimentation relative to the conservative tracer after 6.7 m of transport (20 days travel time) at site F347. Because interactions with grain surfaces can interfere with determinations of sedimentation velocity, only the unretarded microorganisms (those traveling at least as fast as the bromide tracer) were considered. For both injection depths, the center of mass for the transported bacteria arrived at the same time and at the same MLS sampling ports as the bromide center of mass. On the other hand, the DAPI-labeled microflagellates had a measurable rate of sedimentation relative to bromide after 3.6 m of transport at site M4-15. Relative to bromide, the microflagellates (2−3 μm) appear to have been sinking at the rate of 6.4×10^{-8} m/s, yielding a calculated range of buoyant densities of 1.017−1.039 g/cm^3.

Discussion

Bacteria. The observation that uncultured, indigenous bacteria do not appear to sink at substantive rates in small-scale injection and recovery experiment performed at site F347 (Table 2) is consistent with the results of a more recent, 13 m in-situ transport experiment also conducted at the Cape Cod study site (*21*). Buoyant-density determinations could not be accurately determined for the smaller (≤1 μm) size

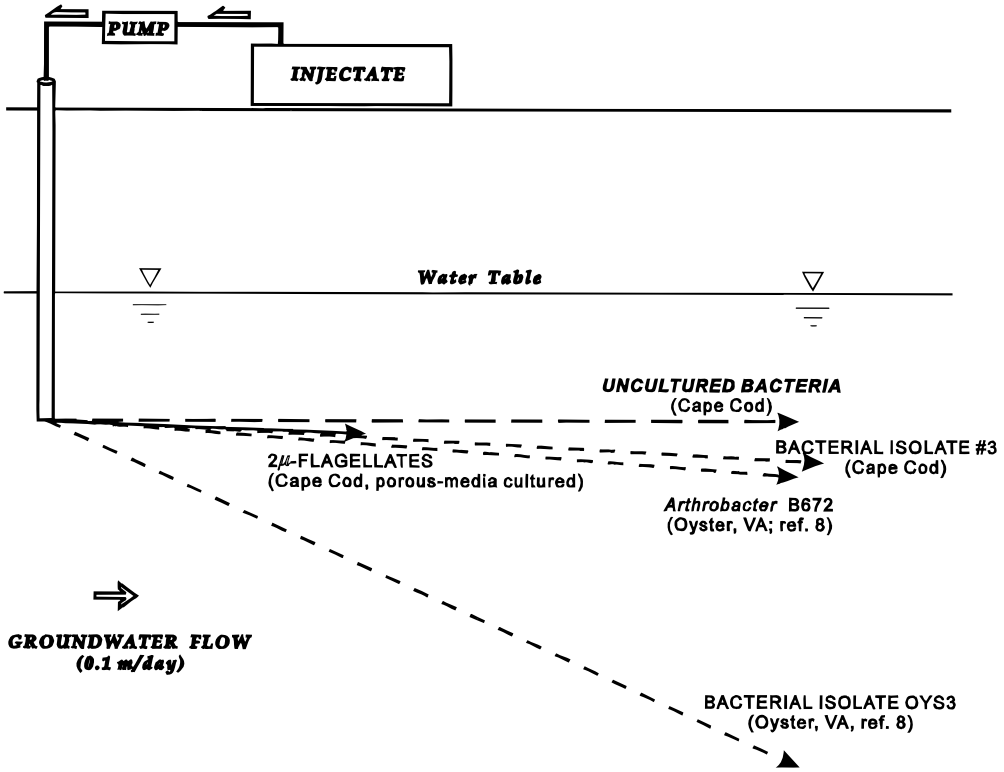

FIGURE 2. Predicted trajectories for uncultured and cultured groundwater microorganisms injected under natural-gradient conditions into a sandy aquifer with a groundwater flow rate of 0.1 m/day. Sedimentation rate for Bacterial isolate 3 (Cape Cod) was estimated using the Stoke's equation and measured size of 1.4 μm and buoyant density of 1.082 g/cm^3. Sedimentation rates for the other microorganisms were measured in saturated sand in the laboratory (*8*) or in the field (this study). Flagellates were assumed to be retarded by a factor of 2.4 relative to the bacteria (*11*).

classes, because their rates of settling would be insufficient to allow vertical separation from the bromide tracer within the experimental time frame, irrespective of buoyant density. However, it may be assumed that the largest (e.g., 2 μm) DAPI-labeled bacteria were near-neutral buoyancy, judging from their close tracking of the bromide tracer. Also, after 20 days of travel, no obvious decrease in the portion of the larger bacteria was observed for the cloud of labeled contaminant plume bacteria.

Our findings that the majority of the bacterial community being advected through the Cape Cod aquifer have DGC-determined buoyant densities < 1.019 g/cm^3 (Table 1) further suggest that most groundwater bacteria seem to be subject to rates of sedimentation that are negligible in comparison to their velocity in the direction of flow. Because of their small size (~0.3 μm) and low buoyant density, the free-living groundwater bacteria inhabiting the pristine zone of groundwater immediately beneath the water table would not be expected to sink at rates fast enough to be easily measured in the field. These findings are consistent with findings for surface water habitats suggesting that small, free-living bacteria do not appear to sink (*22*, *23*). Similarly, the average (0.6 μm) size class of bacteria (buoyant density of < 1.019 g/cm^3) inhabiting the contaminant plume would sink only a few centimeters during the course of 1 year. However, in the absence of interactions with grain surfaces, these bacteria would be advected downgradient more than 100 m during the same period. In general, sedimentation appears to have a minimal effect on the ability of indigenous bacteria to be co-transported with the mobile organic contaminants they are degrading within the plume. Conversely, the frequency and nature of interactions with grain surfaces appear to be the major control of a groundwater bacterium's transport behavior.

The appearance in the DGC tube of a small band of contaminant plume bacteria corresponding to a buoyant density of 1.076 g/cm^3 suggests that there is a minor component (estimated at ~20%) of the free-living community for which settling may need to be considered when predicting their long-term transport behavior. Detailed investigations into the nature of these "heavier" bacteria are beyond the scope of this study. However, it appears they may be largely confined to the contaminant plume, judging from their scarcity in pristine groundwater. Image analysis reveals that the bacteria comprising the heavier fraction of the plume community have an average size of 0.9 μm, which is about ~1.5 times the average size for the bacteria constituting the lighter fraction. On the basis of their size and buoyant density, the heavier bacteria would predictably settle out during large-scale injection and recovery experiments. The calculated sedimentation rates for the heavier bacteria are on the order of ~1 m/year, which is still modest in comparison to the rates of taxis (movement by means of flagella) generally reported for aquatic bacteria. Although the presence of fine grains in aquifer sediments can sharply attenuate a bacterium's random motility coefficient (*24*), migration rates up to 0.1 m/day have nevertheless been observed for motile, non-indigenous bacteria moving through stagnant, nutrient-amended cores of subsurface material (*25*). However, more information is needed about the motility characteristics of groundwater bacteria before such comparisons can be made.

Although sedimentation can be ignored in predictions of subsurface transport for many uncultured groundwater bacteria, cultured groundwater bacteria may exhibit a measurable degree of sinking (Figure 2). A bacterium (OYS3) isolated from an aquifer in Oyster, VA, and grown in LB sunk ~4.3 cm/day in saturated quartz sand under hydrostatic conditions (*8*). The well-known increase in cell size that can occur when a bacterium is isolated from a low-nutrient environment and grown in a LB medium (*26*) is particularly important to its subsurface transport potential. This is because its potential sedimentation velocity predictably

increases as the square of its increase in size (eq 2). Photomicrographs of the fast-settling cultured isolate (OYS3) suggests a large cell length, i.e., 3−5 μm (*8*). Similarly, growth of isolate 1 in LB resulted in an average cell length that was over 4-fold longer than that of the dwarf (0.3 μm) community of groundwater bacteria from which it was isolated (Table 1). This apparent increase in size coupled with an increase in buoyant density (from ~1.01 to 1.04 g/cm^3) would result in at least a 64-fold increase in predicted sedimentation velocity. The range of DGC-determined buoyant densities of the three cultured bacterial strains isolated from Cape Cod groundwater (1.043−1.088 g/cm^3) is similar to the range of 1.040−1.091 g/cm^3 reported for eight LB-grown strains isolated from groundwater in Oyster, VA (*8*). In the latter study, the buoyant densities for the 17 other LB-grown subsurface isolates ranged from 1.06 to 1.12 g/cm^3, suggesting that cultured groundwater bacteria may have buoyant densities comparable to those reported for cultured, non-indigenous bacteria (*6, 7, 27*).

It should be noted that buoyant densities determined from rates of sinking are only rough estimates. In general, the Stoke's equation tends to overpredict the rates of microbial sedimentation (*28*). Differences between observed and predicted sedimentation rates can be due to cell physiology (*29*), surface properties (*30*), and turbulence (*28*). Although the latter factor is not applicable to groundwater environments, the observations that healthy microorganisms are subject to lower sedimentation velocities than less healthy ones (*29*) and that microbial surface properties (surface-associated polymers, electrical charge, and hydrophobic moieties) can substantively affect the dynamic viscosity (eq 2) (*30, 31*) may need to be considered. Deviation from spherical morphology, for which the Stoke's equation is based, and reversible interactions with grain surfaces would also result in differences between observed and predicted sedimentation rates in sandy aquifers. However, good agreement between observed and predicted rates of sedimentation have been observed in the laboratory for at least some cultured groundwater bacteria in porous media (*8*). The best agreement between predicted and observed sedimentation rates in groundwater would be expected for large, healthy, coccoidal bacteria with smooth surfaces and low propensities for interactions with grain surfaces.

The use of the Stoke's equation and buoyant densities ≥1.04 g/cm^3 may be appropriate to describe the initial rate of sedimentation that would occur when bacteria grown in LB are introduced into an aquifer. However, at least some adaptation (or re-adaptation) to in-situ conditions must occur for long-term survival. Adaptation (or re-adaptation) to extreme nutrient limitation could affect microbial size, buoyant density, morphology, physiology, and (or) surface characteristics (*32*−*34*), all of which affect sedimentation rate in granular aquifers. The time frames required for a cultured groundwater bacterium to exhibit physical or compositional changes relating to its re-adaptation to aquifer conditions are not known, but a period of days would not be unreasonable. In a batch growth experiment, a cultured groundwater bacterium took less than 5 h to exhibit 6- and 3-fold decreases in DNA and total adenylate content, respectively, in response to a 10-fold decrease in growth rate (*35*). In another batch experiment, a bacterium isolated from oil well water underwent a 0.25-μm decrease in average cell size in response to starvation over a 24-day period (*36*). For groundwater microflagellates isolated from aquifer sediments, cultured in high-nutrient LB, and reintroduced into an aquifer, decreases in cell size and propensity for grain surfaces were observed in less than a week (*11*). Such physical changes would also affect sedimentation rate. Therefore, it appears that caution should be used when invoking the Stoke's equation and high values of buoyant density to predict long-term settling for cultured bacteria injected into aquifers. This has important implications for engineered restoration of contaminated aquifers, particularly when settling rates are heavily factored into the predicted dispersal rate of an introduced species.

Microflagellates. Because of their size (2−3 μm), sedimentation rates for the groundwater microflagellates would be expected to be much greater than those for the indigenous unattached bacteria (average size 0.3 μm) that presumably constitute a food source. In the 2−3-μm size range, buoyant density can be an important determinant of transport behavior. However, the buoyant density for microflagellates cultured in porous-media conditions was only slightly higher than that of the uncultured bacteria, judging from results from the DGC assay and from the in-situ sedimentation rates. The effect of culturing upon the apparent buoyant density of these organisms is not fully known. However, the lower nutrient, low-pH porous-media growth procedure seemed to preserve the flagellate's cell size distribution (2−3 μm). Also, the transport behavior of microflagellates harvest from the interstitial spaces of the porous growth medium resembled that of vitally-stained microflagellates cultured in high-nutrient liquid media, but allowed to re-adapt to aquifer conditions for several weeks (*11*). According to filtration theory (*2*) and to experimental results involving protozoa-sized microspheres (*11*), the smaller of the indigenous groundwater microflagellates in Cape Cod may be near the optimal size for transport through aquifer sediments. Results from the 1994 injection and recovery experiments suggest a substantive transport potential for the porous-media-grown microflagellates (*37*). This transport potential is facilitated by their small size and low buoyant density relative to most other aquatic protists. Even though the groundwater microflagellates in the Cape Cod aquifer appear to associate largely with particle surfaces (*15*), their potential for transport may allow redistribution within the contaminant plume in response to changes in organic loading. This possibility may have important ramification for aquifer restoration and is worthy of further study.

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