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Tracking of injected and resident (previously injected) bacterial cells in groundwater using ferrographic capture

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Abstract

A high-resolution bacterial tracking technique, ferrographic capture, was used to enumerate fluorescent-stained bacterial cells that were injected into groundwater during a field experiment. The goal of the experiment was to investigate whether detachment of previously injected stained resident cells attached to aquifer sediment was enhanced in the presence of the newly injected mobile cells. This injection was an improvement on past experiments in that the attached (resident) cells were stained, allowing their concentrations to be enumerated directly by ferrographic capture (upon detachment). Contrary to expectations based on previous experiments, enhanced detachment of stained resident cells did not occur upon the arrival of injected cells. Consistent with previous experiments, however, was the observation of ephemeral increases in unstained cell concentrations coincident with the arrival of the stained injected cells. The ephemeral pulses of unstained cells were previously speculated to represent enhanced detachment of unstained indigenous cells in response to hydrodynamic collision with injected cells. The lack of enhanced detachment of stained resident cells in the present experiments indicates that increased concentrations of unstained cells may have occurred by mechanisms other than hydrodynamic collision. Visually observed variations in stain intensity indicated that increased unstained cell concentrations may have resulted from cell division at the low-concentration fringe of the injected plume.

Keywords: Ferrographic capture; Bacterial cells; Hydrodynamic collision

1. Introduction

Bacterial attachment and detachment during transport in porous media is a subject of interest to those concerned with filtration for water treatment (Harvey and Garabedian, 1991), pathogen transport in groundwater (Harvey, 1997), and in situ bioaugmentation (Steffan et al., 1999). Bacteria are colloidal, being in the micrometer size range, and despite their greater complexity, their transport is governed by many of the mechanisms that govern the transport of mineral colloids. Mechanisms controlling colloid detachment have been less studied relative to those governing attachment, likely because rates of colloidal detachment tend to be orders of magnitude lower than rates of attachment. The large difference in rates of attachment versus detachment allows the exclusion of detachment in models describing the gross transport behavior of colloids (e.g. filtration theory). However, second-order aspects of colloid transport are con-

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trolled or influenced by detachment, e.g. bacterial tailing following an input pulse (Johnson et al., 1995; Zhang et al., 2001), and the profile of attached cell concentrations versus distance (Zhang et al., 2001). Among the many processes potentially influencing colloidal detachment is hydrodynamic collision between mobile and attached colloids, which has been observed for microspheres in impinging jet flow experiments (Dabros, 1989; Dabros and van de Ven, 1992) and bacteria and microspheres in parallel plate chambers (Meinders et al., 1995).

That hydrodynamic collision between mobile and attached particles can enhance detachment of attached particles has been established based on force balance calculations (Dabros, 1989; Dabros and van de Ven, 1992), which indicate that hydrodynamic interaction between deposited and flowing particles in stagnation point flow systems causes significant variations of the normal force acting on the deposited particles even for relatively large particle separations, resulting in the escape of weakly bound particles. Bacteria are relatively "soft" particles, which thereby decreases their potential for hydrodynamic collision relative to "hard" mineral colloids. However, after observing increased detachment of attached microspheres and attached bacteria in the presence relative to the absence of mobile bacteria or microspheres, Meinders et al. (1995) concluded that hydrodynamic collision operated in their experiments in impinging jet flow systems.

Tentative observation of enhanced detachment of attached cells in the presence of mobile cells was recently reported from a bacterial transport study conducted in Oyster, VA (Johnson et al., 2001). In this past study (conducted July 2000), two strains of bacteria were injected that had been previously isolated from the subsurface of the Oyster site (DeFlaun et al., 1990). The isolates were stained with a vital fluorescent stain (Fuller et al., 2000) and were injected into established flow cells at the site. Injected cells were distinguished from resident cells (unstained indigenous cells) on the basis of their internal stain. Ephemeral increases in concentrations of unstained bacteria coincident with arrival of the stained bacteria were observed in several wells during the transport experiment. The unstained cells could have originated from either the injected cell population or the population indigenous to the aquifer (not injected). To originate from the injected cell population, the unstained cells would need to have been selectively concentrated relative to stained cells during transport. This could have occurred by division of injected cells (assuming lack of stain transfer to daughter cells), loss of stain by diffusion during transport, and lesser adhesion of unstained relative to stained cells. Cell division and stain loss in samples collected during peak breakthrough were insufficient to explain the observed pulses of unstained cells. Standard adhesion assays indicated no difference in adhesion of stained versus unstained cells. Furthermore, to explain the observed ephemeral dominance of unstained cells, selective concentration would need to have occurred exclusively on the low-concentration fringes of the bacterial plume. Based on the above observation, it was tentatively concluded that the unstained cells originated from the cell population indigenous to the aquifer (not injected). Potential mechanisms of appearance of unstained indigenous cells include growth or detachment in response to the arrival of injected cells, with detachment more likely, given the lack of a lag time between arrival of the unstained and injected cells.

A significant drawback of the previous study was reliance on indirect means to quantify unstained cells, i.e. unstained cells were quantified by the difference between stained and total cells (stained plus unstained). In the present study, potential enhanced detachment of cells from the site sediment, in response to the arrival of mobile injected cells, was monitored directly since the attached cells were stained. It was determined in July 2001 that previously injected cells (remaining from the July 2000 injection) remained intact and visibly stained in the site groundwater, and it was assumed that these cells also resided on the sediment. In July 2001, the same two bacterial strains were injected as in the previous year, however, the cells were stained oppositely from the cells injected in the previous year (the July 2000 injection). Switching the stains allowed for direct observation of the stained cells remaining from the July 2000 injection (hereafter referred to as stained resident cells).

Detachment of attached cells by hydrodynamic collision with mobile cells is thought to require generation of sufficient normal force to remove the attached cells. The magnitude of the normal force

resulting from hydrodynamic collision is directly proportional to the rate of shear due to flow in the system (Dabros and van de Ven, 1992). In calculations described by Dabros (1989) and Dabros and van de Ven (1992), the wall shear rates were in the range of 100–1000 s^{-1}. In the experiments performed by Meinders et al. (1995), which examined rates of microsphere and bacteria detachment, the shear rates were ~ 50 s^{-1}. The shear rates in our experimental systems are calculated to be about 5–10 s^{-1}, based on idealized parabolic pore geometry (Bergendahl and Grasso, 2000) and a representative grain size of 300 μm. These calculated values are 5–10 times less than those examined by the above-described studies (Dabros, 1989; Dabros and van de Ven, 1992; Meinders et al., 1995). However, in our system of packed porous media, the calculated values of shear likely underestimate the actual values for several reasons: (1) the grain size is distributed, thereby decreasing the pore throat sizes below those expected for homogenous media that comprised of 300-μm grains; (2) the grains are not smooth nor spherical, and thereby do not result in the parabolic pore geometry idealized for our calculation.

Because the hypothesized collisions are hydrodynamic rather than direct, it may not be appropriate to assess the potential rate of hydrodynamic collision based on well-known rates of direct collision based on filtration theory (e.g. Ryan and Elimelech, 1996). However, given the above caveat, the rates of direct collision (per meter of transport) based on filtration theory were 60 and 850 for assumed average grain sizes of 500 and 100 μm, respectively. A range is given since the grain size of the natural sediment is distributed (the sediment displays an arithmetic mean grain size of 270 μm). The ranges in collision number obviously do not account for the packing effects of distributed grain sizes, but give some idea of the potential frequency of bacterial collision with sediment grain surfaces. The unstained cell concentrations in the pulses observed in the previous experiment typically ranged from several hundred to several thousand cells per milliliter, which appears to be supportable via collision numbers between 60 and 850 (per mobile cell). However, it must be stressed that the utility of the collision number in assessing the feasibility of hydrodynamic collision is limited, since the "collisions" that are hypothesized to occur between mobile and attached bacteria are hydrodynamic rather than direct.

There is no simple means known to the authors of accurately determining the values of shear or the potential frequency of hydrodynamic collision in a packed porous media. Hence, the opportunity of a direct test for enhanced detachment of resident cells was useful. If enhanced detachment of resident stained bacteria had been observed, then further investigation of its causes would have been warranted. In contrast, the observed lack of enhanced detachment reported below indicates the need to further consider other mechanisms of appearance of the unstained cells. Regardless of outcome, the investigation described below demonstrates the utility of the high-resolution technique that was used for tracking the bacteria.

2. Methods

Two bacterial strains were originally isolated from the site and were used in this study. DA001 is an aerobic adhesion-deficient variant selected using column assays, and is identified as a *Comamonas* sp. (DeFlaun et al., 1990). DA001 is Gram-negative and is 1.2 × 0.6 μm in size. OY-107 is a facultative iron-reducing bacterium of the genus *Acidovorax* that was determined to be naturally adhesion deficient upon isolation from the South Oyster (SO) site. OY-107 is a Gram-negative bacterium of size 1.9 × 1.0 μm.

Bacterial strains were grown by Envirogen (Lawrenceville, NJ) in minimal media supplemented with lactate using standard fermentation procedures. Strains were harvested by centrifugation and starved in at a cell concentration of approximately 10^9 cells/ml in artificial site groundwater for at least 48 h (DeFlaun et al., 2001). Cells were stained during preparation by Envirogen using vital fluorescent stains. Vital fluorescent stains cause no adverse effect to cell culturability (Fuller et al., 2001). DA001 was stained using the green fluorescent stain 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFDA/SE). OY-107 was stained using the red fluorescent stain 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester (TAMRA/SE). Immediately prior to injection, cell suspensions were diluted into site groundwater to a nominal concentration of 1.5× 10^8 cells/ml.

Bacterial cell counts were monitored using ferrographic capture, which combines the selectivity of immunomagnetic tagging with the high resolution of ferrography. For the ferrographic capture analyses, polyclonal rabbit antibodies (Rockland Immunochemicals, Gilbertsville, PA) raised to whole cells of the target bacterial strains were used to tether goat anti-rabbit-coated paramagnetic beads (50-nm diameter, Miltenyi Biotec, Auburn, CA) to the surface of the target cells following sample collection (Zhang and Johnson, 1999; Zhang et al., 1999). The bacterium–bead suspension was introduced into a Bio-Ferrograph (Guilfoyle, Belmont, MA), which deposited the magnetically tagged bacteria onto a small area on a glass substratum. The bacteria were then enumerated under an epifluorescence microscope. TAMRA/SE- and CFDA/SE-stained cells were enumerated using red (λ_{ex} = 510–560 nm, dichroic—575 nm, λ_{em} = 590 nm) and green (λ_{ex} = 470–490 nm, dichroic—505 nm, λ_{em} = 510–530 nm) filters, respectively. Previous studies have shown that ferrographic capture provides virtually 100% recovery of target cells and yields extremely high analytical resolution (~ 20 cells/ml) (Zhang and Johnson, 1999; Zhang et al., 1999, 2001; Johnson et al., 2000, 2001).

The same two bacterial strains were used in the year 2000 and year 2001 injections; however, the stains were switched between the two strains for the July 2001 experiment relative to the July 2000 experiment (Table 1). Hence, in groundwater samples, the two different strains were distinguished antigenically, whereas the injection year was distinguished using the internal stain. Unstained (or weakly stained) cells were also monitored using anti-DA001 and anti-OY-107 antibodies that had been conjugated to a green fluorophore, fluorescein isothiocyanate (FITC).

Since the FITC-conjugated antibodies provide a visible outline of both unstained and stained target cells, the unstained cell concentrations were determined by difference between analyses using FITC-conjugated antibodies (stained plus unstained cells) and nonconjugated antibodies (stained cells). The antibodies may have also captured antigenically similar nontarget cells. Although nontarget cells would not be enumerated using nonconjugated antibodies (in analyses for stained cells), they would be enumerated using FITC-conjugated antibodies (in analyses for stained plus unstained cells). Hence, the "unstained" cell counts may include both target strains as well as antigenically similar cells. Laboratory tests showed that the antibodies successfully capture wild-type DA001 and wild-type OY-107 cells (where wild type refers to initial isolates), indicating that the analyses could indeed be capable of capturing indigenous forms of these strains.

Table 1
Stains used during the series of experiments

Year	DA001	OY-107
2000	TAMRA/SE	CFDA/SE
2001	CFDA/SE	TAMRA/SE

Since both bacterial strains were potentially present in both colors in each groundwater sample, the extent of antibody cross-reactivity between the two bacterial strains was determined. Serial dilutions of both bacterial strains collected from their respective injection lines were analyzed using the respective nontarget antibody. It was determined that approximately 1% of cells from serial dilutions of both bacterial strains reacted with their respective nontarget antibody (data not shown). However, unstained cell suspensions grown and serially diluted in the laboratory showed zero cross-reactivity with their nontarget antibody (data not shown), indicating that the apparent 1% cross-reactivity observed in the injection line standards may have resulted from cross-contamination during sampling of the injection lines. Even 1% apparent cross-reactivity would have insignificant effect on the bulk of our analyses, since cell concentrations examined by ferrographic capture were primarily in the 10–1000 cells/ml range, yielding a maximum of 10 cells/ml nontarget cells in the samples. However, interpretation of results from samples containing greater than 1000 cells/ml necessarily considered potential effects of cross-reactivity.

Another potential artifact, given the possibility that each of the two strains could be present in each of the two colors, was the potential visibility of one stain under the filter used for the other stain during enumeration. To avoid this problem, filters were switched back and forth during enumeration to ensure that the cells being counted were exclusively observed under the target filter.

Samples taken from the injection solutions were collected to serve as standards (following dilution to

1000 cells/ml) for ferrographic analysis. These standards and blanks consisting of high purity Milli-Q water were included in each set of eight samples analyzed. Standard concentrations of both bacterial strains were observed to degrade at approximately 1% per day in all ferrographic analyses (using FITC-conjugated and nonconjugated antibodies). The decrease in captured cell number over time is likely due to antigen alteration in the presence of formaldehyde, as has been previously reported (Johnson et al., 2000).

Flow cytometry (FACScan, Becton-Dickinson Immunocytometry Systems, San Jose, CA) was used to monitor cell concentrations of CFDA/SE-stained DA001 when they were present at concentrations greater than 5000 cells/ml. Although ferrographic capture can analyze cell concentrations above this magnitude following 1:10 or greater dilution, flow cytometry has the advantage of being extremely fast. A filter to allow detection of TAMRA/SE-stained OY-107 was not available on the instrument, and so TAMRA/SE-stained cells were enumerated exclusively using ferrographic capture. TransFluoSpheres (Molecular Probes, Eugene, OR) were added at a known concentration (60,000 spheres/ml) to the samples in order to determine volume of sample analyzed. Each sample was run for 50 s at the low flow rate setting of the machine, which corresponds to approximately 10 μl/min.

3. Field experiments

The South Oyster (SO) focus area is one of two flow cells located at the South Oyster site in Oyster, VA on the southern end of the Delmarva Peninsula. The SO flow cell is located in a surficial aquifer that comprised of unconsolidated to weakly cemented, well-sorted, medium- to fine-grained sands and pebbly sands. The aquifer is underlain by a fine-grained muddy silt aquitard situated approximately 9 m below ground surface (BGS). A peat layer from ~ 2.5 to 4.0 m BGS lies within the granular sands. The SO focus area flow cell is bounded vertically by the peat layer above and the clay aquitard below. The sediment between the peat layer and the aquitard is dominantly very fine sand and fine sand (quartz, feldspar, and significant micaceous minerals) with occasional medium- and coarse-grained sand stringers. The SO flow cell is characterized as suboxic, with dissolved oxygen concentrations in the range of 0.2–1.0 mg/l.

Twenty-four multilevel samplers (MLS) exist in the flow cell (Fig. 1) in an array that is oriented with the main axis parallel to natural local groundwater flow direction, as inferred from water level observations prior to site installation. The flow cell is bounded at the up-gradient limit by the injection well SO-B2 and at the down-gradient limits by three extraction wells SO-A3, SO-B3, and SO-C3, which form a line perpendicular to flow about 19.5 m down-gradient of SO-B2.

Each MLS consisted of a 3-cm-diameter PVC rod separated into 12 equally spaced zones that is separated by neoprene baffles. Stiff polyethylene tubing (3.2 mm ID) extended from approximately 0.5 m above ground surface to each sampling port. Detailed descriptions of the MLS used at SO focus area can be found elsewhere (Mailloux et al., in press). In this paper, the MLS are denoted by their number and the prefix "SO", e.g. SO-17 denotes South Oyster focus area MLS 17. The sampling port is denoted by

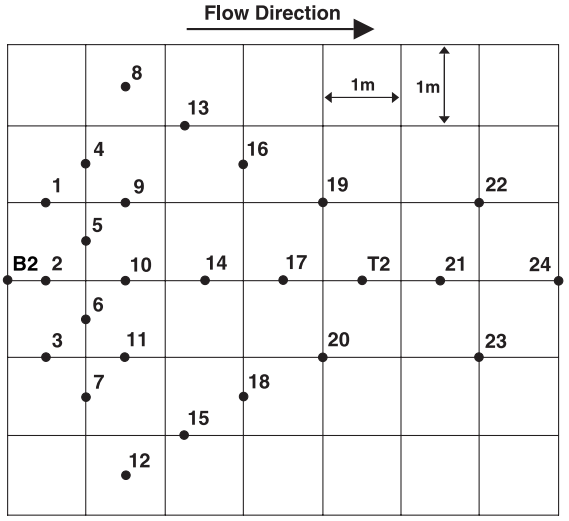

Fig. 1. Layout of the South Oyster focus area flow cell. Numbers refer to multilevel samplers (MLS). B2 is the injection MLS. The forced gradient was set 1 week prior to injection by extraction from three wells in a line oriented perpendicular to flow 12 m down-gradient of MLS SO-24.

number (1–12) and the prefix "P", e.g. SO-17 P4 denotes port 4 in MLS 17. The port number increases with depth in the flow cell.

The ambient pore water velocity at the site is approximately 0.1 m/day. Forced gradient conditions were established at the site approximately 48 h prior to the start of injection by withdrawing from the three down-gradient extraction wells, SO-A3, SO-B3, and SO-C3, at flow rates of 20, 40, and 20 ml/min, respectively. The resulting average pore water velocity was approximately 1 m/day.

Injection was conducted in MLS SO-B2, a 4-in. PVC well with four vertically discrete injection zones separated by packers. In contrast to the previous experiment (July 2000), in which both bacterial strains were injected over the entire depth of the flow cell, the July 2001 experiment injected the two bacterial strains over two discreet depths. DA001 was injected over the interval of −3.5 to −4.5 m relative to mean sea level (MSL), and OY-107 was injected over the interval of −5.0 to −6.0 m MSL, both at injection rate of 0.8 l/min. The bacterial injection zones were separated by injection of unamended groundwater into the interval from −4.5 to −5.0 m MSL at a rate of 0.4 l/min. Unamended groundwater was also injected above the DA001 injection zone (−3.0 to −3.5 m MSL) at a rate of 0.4 l/min. The total injection rate from SO-B2 was 2.5 l/min. Injection occurred over a 12-h period. Both bacterial injection solutions were sampled every 30 min during the injection interval.

A custom manifold was developed to apply continuous suction to all sampled ports simultaneously using a peristaltic pump (Johnson et al., 2000; Mailloux et al., in press). All sampled ports were pumped continuously and equally in order to minimize inducement of a vertical hydraulic gradient between the ports. Relative to the previous experiment (July 2000), the number of samples taken during early breakthrough was increased in the July 2001 experiment to increase the resolution of the early breakthrough pulses. All bacterial samples were taken using 50-ml polypropylene centrifuge tubes and then fixed with 1% formaldehyde. Samples were then split into smaller aliquots (15 ml), briefly stored on ice, shipped back to the University of Utah on ice (unfrozen), and stored at 4 °C.

4. Laboratory experiments

Several laboratory experiments were run to investigate potential enhanced detachment of attached cells in a controlled system. Each experiment involved a loading step, in which a bacterial suspension was introduced to a sediment-packed column. Loading was followed by elution until the effluent cell concentration was 100 cells/ml or less. Elution was followed by injection of a cell suspension to determine whether detachment of previously loaded (resident) cells was enhanced by arrival of mobile cells.

Sediments used in the column experiments were obtained from the narrow channel (NC) focus area in Oyster, VA. The sediment is primarily composed of quartz, feldspar, clays, and iron and aluminum hydroxides in decreasing order of abundance (Johnson et al., 2000). A target average pore water velocity of 1 m/day for each experiment was maintained throughout the duration of the experiment. Flow was allowed to equilibrate at the target average pore water velocity for at least 1 pore volume (PV) prior to injection of the bacterial solutions. Narrow channel artificial groundwater (NCAGW) was used as the aqueous medium in all laboratory transport experiments (DeFlaun et al., 2001).

Strains were grown according to growth protocols described earlier (DeFlaun et al., 2001). Following collection, all samples were fixed with 1% formaldehyde. Since the concentrations of attached resident cells in the field experiments were not known, it was not clear what attached bacterial concentrations would be reflective of field conditions. For this reason, injection duration (0.2–0.5 pore volumes), injection concentration (2×10^3 to 4×10^5 cells/ml), and column size (2.5 cm diameter by 15 cm length to 7 cm diameter by 53 cm length) were varied among the set of column experiments to yield resident (attached) cell concentrations ranging from about 50 to about 1.5×10^5 cells per gram of sediment. The concentration of subsequently injected bacteria was also varied (2×10^3 to 4×10^5 cells/ml).

5. Results

MLS SO-10 and SO-14 were sampled in July 2001, prior to the establishment of forced gradient

conditions at the site, in order to determine the stained resident cell concentrations (remaining from July 2000 injection). Stained resident cells of both strains remaining from the July 2000 injection were observed in both sampled MLS. TAMRA/SE-stained DA001 concentrations were approximately 50 cells/ml at all depths in SO-10, and were approximately 200 cells/ml at all depths in SO-14. CFDA/SE-stained resident OY-107 cells were observed at low concentrations (averaging ~ 20 cells/ml) at all depths in SO-10 and low concentrations (averaging ~ 30 cells/ml) at all depths in SO-14. Laboratory blanks showed no cells, proving that the cells did not derive from contamination during analysis. Tubing in all MLS was removed and replaced prior to monitoring for the year 2001 injection, eliminating the possibility that these cells had remained in the tubing during the year that passed between experiments.

No enhanced detachment of stained resident cells was observed to occur with the arrival of injected cells during the July 2001 injection. This is illustrated in several sampling ports in various MLS, including SO-T2 P12, SO-17 P10, SO-24 P8, and SO-24 P12 (Fig. 2), where breakthrough of injected DA001 at 70 (SO-T2 P12), 35 (SO-17 P10), and 160 (SO-24 P8 and P12) elapsed hours was not accompanied by increases in stained resident cell concentrations. SO-T2 P12 shows an example of the background concentrations of stained resident cells in samples taken prior to breakthrough of injected cells. The background concentrations of stained resident cells were not observed in all of the MLS, including three of the four MLS shown in Fig. 2. Results from four other monitored MLS (not shown) also showed a lack of enhanced detachment of stained resident cells regardless of presence of stained resident cells, indicating that enhanced detachment of stained resident cells did not occur in response to breakthrough of injected cells.

It should be noted that the analytical resolution of the analysis allowed enumeration of extremely low cell concentrations during initial breakthrough, and that variations among concentrations during initial breakthrough represent a combination of transport effects due to aquifer heterogeneity, sampling errors,

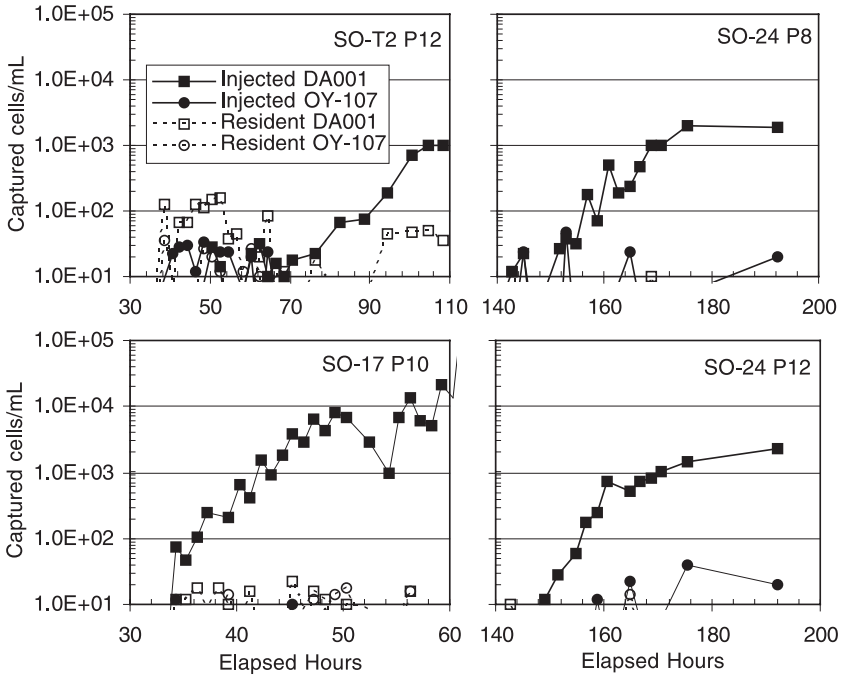

Fig. 2. Results from July 2001 field experiment showing breakthrough of injected DA001 and OY-107. Detachment of resident DA001 and OY-107 cells was not enhanced by breakthrough of the injected cells. Resident DA001 was observed in the aqueous phase before, during, and after extraction (e.g. SO-T2 P12).

and analytical error. In addition to high resolution, the method provided sufficient flexibility to allow simultaneous monitoring of two strains each of two different colors (each of two different injection times) as well as their unstained counterparts in combination with any antigenically similar strains.

Unstained cell concentrations showed ephemeral increases coincident with the arrival of stained injected cells, similarly to increases previously reported from the July 2000 injection (Johnson et al., 2001). MLS SO-17 P2 (Fig. 3) shows, in log (left) and linear (right) format, ephemeral increases of total (injected plus unstained) cells. These ephemeral increases were a factor of three to four times greater than injected cell concentrations in pulses centered at 70, 98, and 120 elapsed hours. MLS SO-17 P4 (Fig. 3) shows ephemeral increases of total cells, a factor of three to four times greater than injected cell concentrations in pulses centered at 106 and 145 elapsed hours.

The unstained cells were observed only in limited locations throughout the site and were exclusively observed in relatively shallow MLS ports (port 4 and above). No significant increases in total relative to injected cell concentrations were observed in the majority of ports analyzed, as shown in MLS SO-17 P6, SO-24 P8, and SO-24 P10 (Fig. 4), where stained and total cell concentrations increased simultaneously with negligible differences (less than a factor of two) upon arrival of injected cells.

Results from the laboratory experiments also indicate that no enhanced detachment of resident cells occurred in response to the arrival of injected cells. Results from a representative experiment are shown in Fig. 5. One pore volume of stained DA001 (2×10^5 cells/ml) was injected into the column (0–2 elapsed hours), resulting in an effluent pulse from 2 to 5 elapsed hours. Extended tailing of low concentrations of stained DA001 was observed during elution with NCAGW. In this column experiment, two separate reservoirs were used for delivery of the bacterial suspension and NCAGW, and switching between the two reservoirs required the use of a

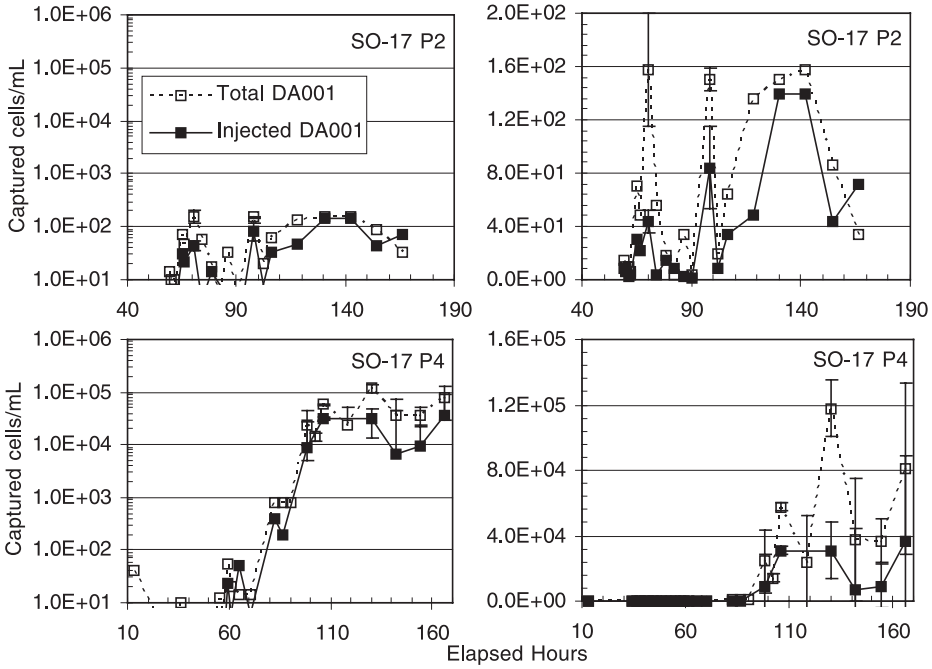

Fig. 3. Results from July 2001 field experiment showing an ephemeral pulse of unstained cells (difference between total and stained cell concentrations) coincident with the breakthrough of injected cells. The peak unstained cell concentrations were more than a factor of two greater than the concentrations of injected cells in several ports. This result was observed only in the shallow ports.

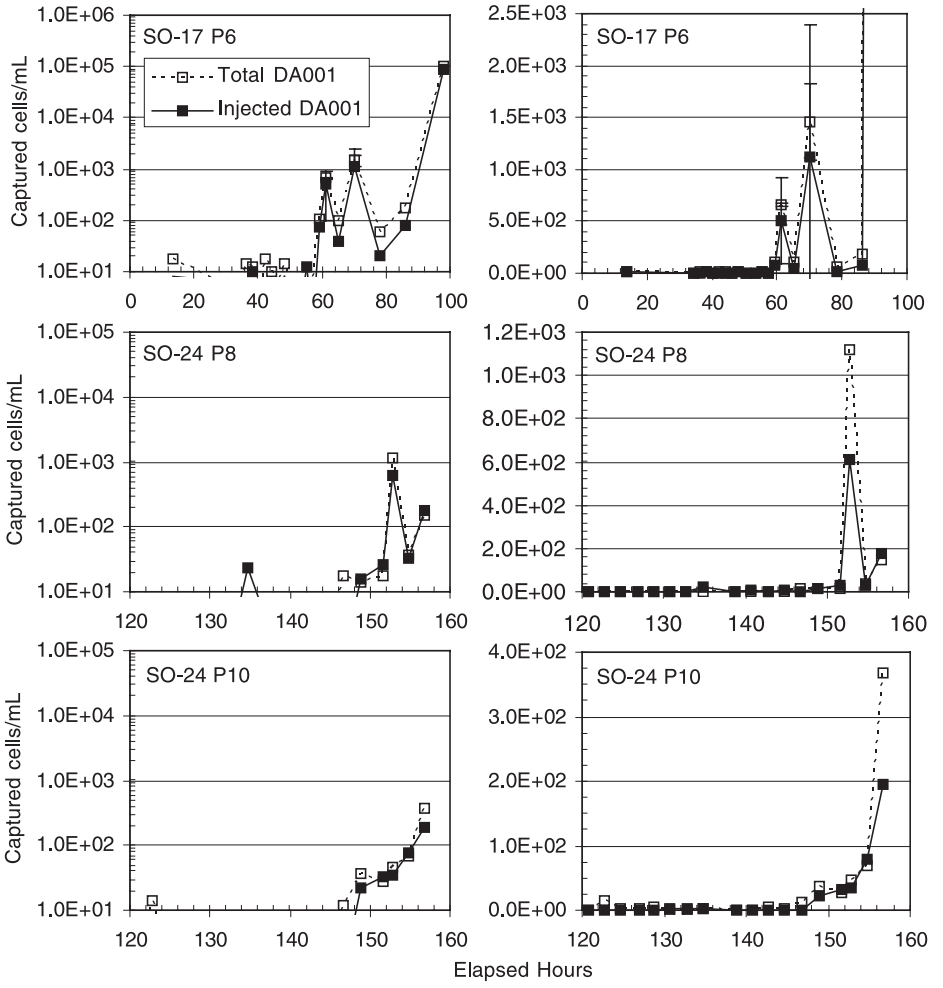

Fig. 4. Results from the July 2001 field experiment highlighting the lack of significant unstained cell concentrations (difference between total and stained cells) in the majority of the MLS ports sampled at the site.

three-way valve. Unlike the field experiment, switching between reservoirs during the laboratory experiment may have caused a sudden increase in hydrodynamic shear in the column. To assess the magnitude of enhanced detachment due to hydrodynamic shear, the three-way valve was quickly switched between reservoirs at about 24 elapsed hours (Fig. 5).

Following the valve switch, a pulse injection of 3.5e5 cells/ml of unstained DA001 cells was then introduced into the column beginning at ~29 elapsed hours. The effluent pulse of stained resident cells at 24–28 elapsed hours (~ 1×10^3 cells/ml) was associated with the valve switch. The effluent pulse of stained resident cells at 30–33 elapsed hours (1×10^2 cells/ml) was associated with the breakthrough of unstained DA001 cells. The fact that greater enhanced detachment of resident cells occurred during the valve switch alone relative to the valve switch plus introduction of mobile cells indicates that the observed enhanced detachment of the resident cells in the laboratory experiment may have resulted from hydrodynamic shear associated with the valve switch, and cannot be attributed to collision with mobile cells. It should be noted that subsequent experiments conducted in a system that

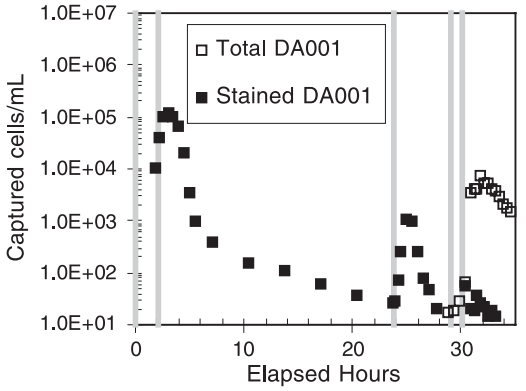

Fig. 5. Representative results from the laboratory column experiments showing a lack of enhanced detachment of stained resident cells. The first two vertical bars represent the time of the injection pulse for stained DA001. The third vertical bar represents the time of the valve switch. The fourth and fifth vertical bars represent the time of the injection pulse of unstained DA001 cells.

avoided valve switching also lacked enhanced detachment of resident cells.

6. Discussion

Enhanced detachment of stained resident cells in response to arrival of injected cells was not observed in the July 2001 field injection, nor was it observed in any of the laboratory experiments. Lack of enhanced detachment of stained resident cells may indicate that the concentrations of stained resident (attached) cells were insufficient to support significant enhanced detachment in the presence of mobile cells, assuming that greater attached cell concentrations increase the magnitude of enhanced detachment. The adhesion-deficient variants used in the experiments may have yielded low attached cell concentrations relative to their indigenous counterparts, although the attached concentrations of the latter are unknown.

Alternatively, the results may indicate that the ephemeral pulses of unstained cells observed during the field experiments represent a process other than enhanced detachment. Mechanisms, other than detachment, that may explain the ephemeral increases in unstained cell concentrations (coincident with the initial breakthrough of injected cells) include preferential stain loss from the injected cell population residing in the low-concentration fringe of the plume (Johnson et al., 2001). Stain loss by diffusion from cells could occur preferentially from cells residing at the low-concentration fringe of the injected plume. However, Fig. 6 shows that CFDA/SE-stained DA001 monitored using FITC-conjugated antibodies (total cells) and nonconjugated antibodies (stained cells) degraded at very similar rates, indicating that stain loss over time was negligible.

Notably, injection-zone samples taken during injection showed concentrations of 1.3×10^8 and 1.2×10^8 cells/ml for DA001 and OY-107, respectively, according to ferrographic capture using FITC-conjugated antibodies. This result agreed well with flow cytometry results for samples collected from the injection tanks, which showed DA001 concentrations of 1.5×10^8 cells/ml. Surprisingly, concentrations of both strains determined ferrographically using non-conjugated antibodies were about ~55% of those determined by the other methods. This latter result was corroborated by direct counts on filters (Mark Fuller, Envirogen, personal communication). These results indicate that nearly 50% of the injected cells were weakly stained. The weakly stained cells in the injection solution may represent inefficiency in the staining procedure (Mark Fuller, Envirogen, personal communication).

The presence of unstained cells in the injected solution does not affect the analysis presented regarding the detachment of stained resident cells. The presence of unstained cells in the injection

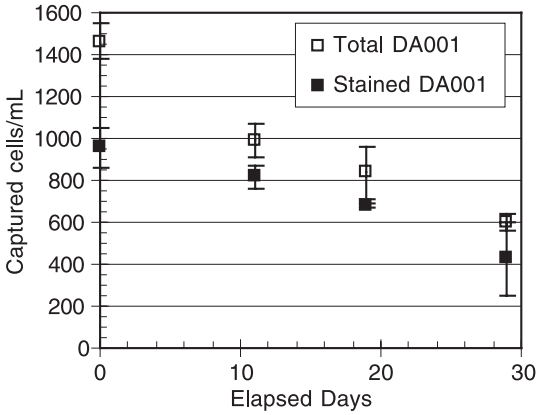

Fig. 6. CFDA/SE-stained DA001 standard results observed using FITC-conjugated antibodies (total DA001) and nonconjugated antibodies (stained DA001) to DA001 cells.

solution does, however, explain the persistent factor-of-two difference between total and unstained cell concentrations observed in nearly all ports when cell concentrations became significant (e.g. greater than 100 cells/ml in Figs. 3 and 4), as well as the factor-of-two difference between standards monitored using FITC-conjugated versus nonconjugated antibodies (Fig. 6). The presence of unstained cells in the injection solution does not, however, explain the ephemeral pulses of unstained cells yielding ratios of total to injected cell concentrations of three to four (Fig. 3).

Stain loss due to cell division at the low-concentration fringe of the plume of injected bacteria represents another potential means of generating the ephemeral pulses of unstained cells. Recall that the ephemeral pulses of unstained cells were observed in relatively shallow ports. Indeed, cells in samples collected from shallow ports exhibited a polar distribution of stain that was suggestive of stain loss due to cell division, in which the daughter cells each retained stain in the portion of the cell that was originally part of the parent cell. Fig. 7 shows two images of polar stained cells (left) and normal fully stained cells (right). The cells that exhibited a polar distribution of stain would easily be missed during enumeration following ferrographic capture by nonconjugated antibodies due to their relatively weak illumination. It is therefore possible that the ephemeral pulses of unstained cells coincident with the injected cells represent stain loss due to cell division in the low-concentration fringe of the plume in the shallow portion of the flow cell.

Cell division in the low-concentration fringe of the plume could potentially have been driven by a favorable combination of electron acceptor and donor exclusively at the interface of the injected and ambient groundwater in the shallow portion of the aquifer. Differences in the chemistries of the injected versus ambient groundwater, if any, are expected to have been subtle, since the injected groundwater was extracted from the site at a location just outside the flow cell. The limited groundwater chemistry data measured during the field injection indicate no significant variation with depth for the measured parameters (including dissolved oxygen, dissolved organic carbon, ionic strength, and pH). The limited data do suggest, however, that dissolved (<0.2 μm) organic carbon concentrations were slightly higher in the ambient relative to the injected groundwater (by 1–10 mg/l), potentially supporting growth at the interface. However, the temporal and spatial sparseness of the chemical data prevent rigorous identification of potential chemical drivers for cell division at the interface between ambient and injected groundwater, and so this determination is beyond the capabilities of the present study.

This investigation highlights the resolution of ferrographic capture, which clearly showed that enhanced detachment of stained resident cells did not occur in response to breakthrough of injected cells in our system. This result weakens the original hypothesis that hydrodynamic collision between mobile and attached bacteria resulted in the observed ephemeral domination by unstained cells during initial breakthrough.

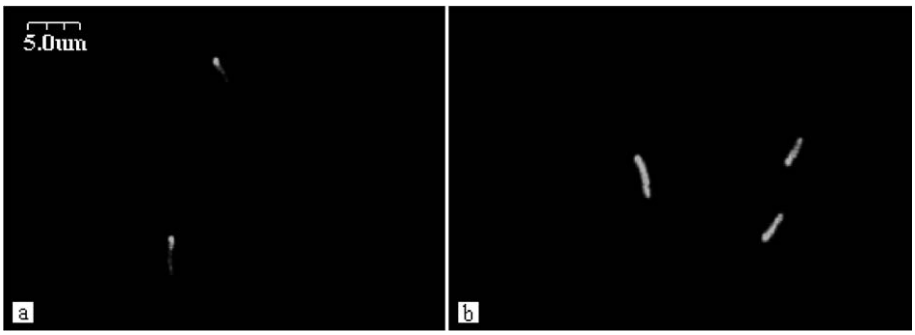

Fig. 7. Images of DA001 cells from the July 2001 field experiment showing polar distribution of stain in relatively dim cells, suggestive of cell division (a). Staining normally results in homogeneously bright cells (b).

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