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FEMS Microbiology Ecology 37 (2001) 55–66

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Field-scale evaluation of CFDA/SE staining coupled with multiple detection methods for assessing the transport of bacteria in situ

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Received 19 February 2001; received in revised form 6 June 2001; accepted 7 June 2001

First published online 28 June 2001

Abstract

This research was undertaken to evaluate staining with the fluorescent compound CFDA/SE (5-(and-6-)-carboxyfluorescein diacetate, succinimidyl ester) coupled with multiple cell detection methodologies as a means to monitor bacterial transport during field-scale experiments. Stained cells of *Comamonas* sp. strain DA001 were injected into a shallow, aerobic aquifer in Oyster, VA, USA. Groundwater samples analyzed in the laboratory using epifluorescence microscopy, flow cytometry, ferrographic separation, and microplate spectrofluorometry yielded comparable concentrations of CFDA/SE-stained DA001 cells, although each method had a different effective lower limit of detection. Determination of cell concentrations in the field using microplate spectrofluorometry allowed the track of the bacterial plume to be monitored in near-real time, but produced results that were not as accurate as laboratory analyses. The CFDA/SE stain was well retained in the cells over a 5-month period. Normal handling of samples under fluorescent and incandescent lighting did not significantly affect sample integrity, but exposure to sunlight resulted in rapid loss of total and per cell fluorescence. The combination of CFDA/SE staining and multiple detection methods was demonstrated as an effective means to study bacterial transport in groundwater. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Bacterial transport; Vital fluorescent staining; Subsurface microbiology; Bioremediation; Microplate spectrofluorometry; Flow cytometry

1. Introduction

The process of bioaugmentation – using degradative microbes to bioremediate soil and groundwater contaminated with recalcitrant pollutants – requires that effective concentrations of microorganisms be predictably transported to contaminated areas of the subsurface. The DOE Natural and Accelerated Bioremediation Research (NABIR) Program is funding extensive research examining the physical, chemical and biological controls on bacterial transport, and a field site has been established in Oyster, VA, USA. One of the key aspects of this research effort is to develop new methods for monitoring the movement of introduced microorganisms, and to evaluate these new methods under both laboratory and field conditions.

One promising approach is based on labeling cells with fluorescent stains, with subsequent detection of the fluorescent cells in groundwater samples. During short-term (∼48 h) laboratory experiments, cells stained with DAPI (4′,6-diamino-2-phenylindole) were shown to be transported similarly to unstained cells through repacked columns of aluminum oxide-coated Ottawa sand, whereas the transport of cells stained with AO (3,6-bis(dimethylamino)acridiunium chloride, 'acridine orange') was retarded and reduced relative to unstained cells [1]. Staining bacteria with DAPI has been employed for several in situ bacterial transport experiments in the Cape Cod aquifer [2–5]. Both DAPI and AO bind to nucleic acids, and are therefore considered to be mutagenic, reducing their applicability for field experiments due to safety concerns. Furthermore, deleterious effects of DAPI on normal cell

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PII: S0168-6496(01)00143-X

function have also been reported [6]. For fluorescent stain-based methods to be useful for studying bacterial transport and monitoring bioaugmentation, the compounds must have minimal effects on bacterial adhesion, viability, and metabolic activity, while at the same time be retained in the cells for at least several weeks and pose minimal threats to the health of humans or the environment.

Previous research has identified a relatively new fluorescent compound, CFDA/SE (5-(and-6-)-carboxyfluorescein diacetate, succinimidyl ester), which was shown to stain bacterial cells uniformly for up to 1 month, with no effects on cell viability or transport through intact sediment cores [7]. CFDA/SE is a derivative of fluorescein, which is considered to pose little threat to human or ecological health, and has been widely used as a dissolved tracer for groundwater testing [8]. CFDA/SE is initially converted to CFSE by intracellular esterases which cleave the acetate moieties from the compound, and the CFSE is then converted to a non-reactive protein conjugate [9]. Methods for quantifying CFDA/SE-stained fluorescent cells using epifluorescence microscopy, microplate spectrofluorometry, and flow cytometry (FC) were also developed [7]. Additionally, an immunomagnetic separation and concentration method known as ferrographic capture has been successfully coupled with CFDA/SE staining to allow enumeration of very low densities of stained cells ($\sim$20 cells ml^{-1}) [10].

The results reported here demonstrate that the CFDA/SE staining technique coupled with several detection methodologies is a rapid and effective means for monitoring field-scale bacterial transport through the subsurface.

2. Materials and methods

2.1. Site description

The Narrow Channel Focus Area (NC) at the NABIR South Oyster field site is situated over the shallow coastal plain Columbia aquifer. The aquifer sediments are predominantly fine to medium grain sands; the groundwater is aerobic (4–7 mg O_2 l^{-1}), contains concentrations of dissolved and total organic carbon of 1–5 mg C l^{-1}, and has a subneutral pH (5.5–6.0 standard units). More complete geological descriptions of the site can be found in Chen et al. [11].

The bacterial transport experiments were performed using the multilevel sampler (MLS) array within the NC flow cell, which is illustrated in Fig. 1. The flow cell is aligned parallel to the natural groundwater flow. Prior to bacterial injection, a forced gradient was established by withdrawing groundwater from extraction wells A3, B3, and C3 (at rates of 10.4, 41.6, and 20.8 l min^{-1}, respectively) to achieve a mean groundwater flow velocity of 1.0 m day^{-1}. A portion of extracted groundwater was re-injected at B2 to achieve a total input of 5.0 l min^{-1}, while the remainder was discharged into a wooded area approximately 100 m southwest of the flow cell. Depth-to-water measurements were taken throughout the experiment to assure that the hydraulic head did not vary by more than 0.02 m perpendicular to the direction of groundwater flow.

The MLS array consisted of 24 MLSs (S1 to S24, Fig.

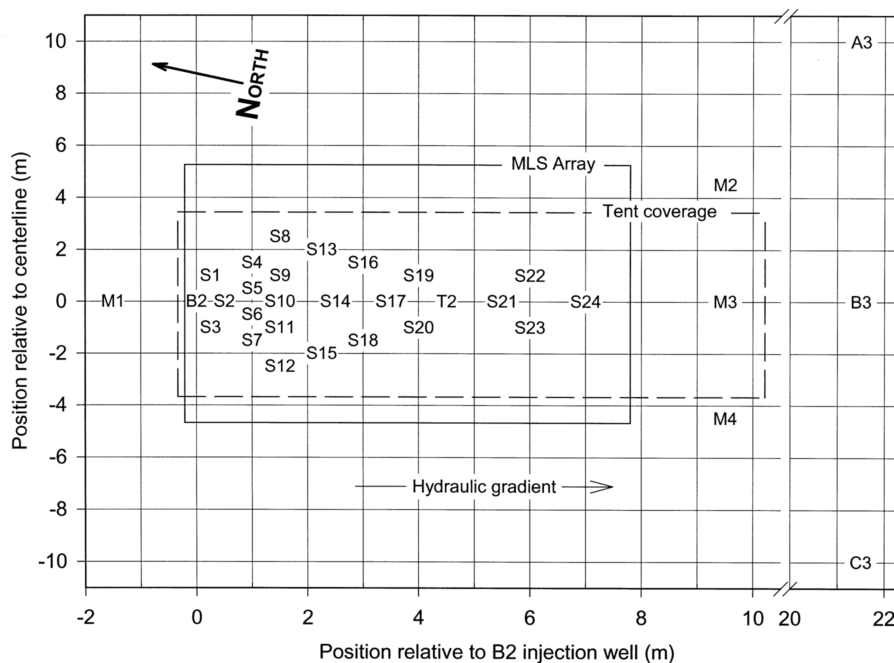

Fig. 1. Layout of the Narrow Channel Focus Area flow cell at the South Oyster site, indicating the position of the extraction wells (A3, B3, C3) and injection well (B2), and direction of the imposed hydraulic gradient. The areal coverage of the tent over the MLS array during the experiment is indicated by the dashed line. The aspect ratio is approximately 1:2 (x-axis to y-axis).

1), each equipped with 12 0.16-m screened ports terminating at discrete depths (P1 to P12); the center of the sampling intervals, expressed as meters below ground surface (m bgs), were as follows: P1, 3.02; P2, 6.38; P3, 6.58; P4, 6.86; P5, 7.14; P6, 7.42; P7, 7.69; P8, 7.97; P9, 8.25; P10, 8.53; P11, 8.81; P12, 9.09. The depths expressed as meters mean sea level (m msl) can be calculated by subtracting each sampling depth from the depth of the ground surface, +3 m msl. The layout of the MLS array within the NC flow cell, and the vertical spacing of the ports was based on pre-experiment modeling results as described elsewhere [12]. The MLSs were attached to specially designed manifolds which allowed groundwater from the different ports to be sampled (see below). The manifolds were designed to allow continuous groundwater withdrawal from each port of the MLS at a rate of approximately 4.0 ml min^{-1}, which did not significantly affect the overall flow pattern.

During the October 1999 bacterial transport experiment, a large tent was used to cover the entire MLS array area, to provide general protection from the environment and prevent exposure of samples to direct sunlight. Manifolds, peristaltic pumps and other equipment were placed on 1-m high tables; a 10-m long recreational vehicle (RV) provided additional workspace. Two 20-kW generators were used to meet the site's electrical needs.

2.2. Chemicals and media

CFDA/SE was purchased from Molecular Probes (Eugene, OR, USA) and Fluka Chemical Company (Ronkonkoma, NY, USA), both at purities of $>95\%$. $^{13}CH_3^{13}COONa$ (99 atm%) was purchased from Isotec, Inc. (Miamisburg, OH, USA). R2A agar was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Artificial groundwater (NCAGW, pH 6.0) was based on the Oyster, VA, USA site groundwater chemistry [7] and sterilized by 0.2-μm filtration. Basal salts medium (BSM) composition was as described by Hareland et al. [13], except that nitrilotriacetic acid was omitted. Phosphate-buffered saline (PBS, pH 7.4) consisted of 8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 l^{-1} of deionized distilled water and was sterilized by filtration.

2.3. Bacterial strains

An adhesion-deficient variant of *Comamonas* sp. strain DA001 was used for this research [7]. The aerobic organism was originally isolated from the Columbia aquifer underlying the NC focus area. Routine growth of the organism was performed using 0.2% (w/v) sodium acetate in BSM at room temperature.

2.4. Bacterial growth and labeling

Growth of large volumes of high densities of DA001 cells was performed in Envirogen's fermentation facility (Lawrenceville, NJ, USA). Preliminary experiments established a growth protocol which achieved a yield of 0.16 g biomass (dry weight) per gram of sodium acetate. Preparation of the field inoculum began with an initial 200-ml culture (0.2% (w/v) sodium acetate in BSM), which was used to inoculate 1.4 l of the same medium. A portion of this culture (1.2 l) was used to inoculate a BL 20.2-l fermenter (LSL Biolafitte, Inc., Minneapolis, MN, USA), operating with a total volume of 13 l at 28°C. Aliquots of filter sterilized sodium acetate (20%, w/v) were added over the course of 72 h. The pH was kept between 6.8 and 7.2 using 5 N NaOH and 6 N H_2SO_4. Dissolved oxygen was kept at saturation by adjusting the air flow and reactor mixing speed. The culture was amended with trace elements and nitrogen (as NH_4Cl) periodically to assure maximum growth. When the culture reached an optical density (OD_{550}) of 10 (approximately 1×10^{10} cells ml^{-1}), it was prepared for CFDA/SE staining.

Scale-up of the CFDA/SE staining method as described in [7] was performed as follows. DA001 cells were harvested by centrifugation (3400 rpm, 30 min, 4°C), washed twice in PBS, and returned to the 20-l bioreactor resuspended in a final volume of 14 l PBS. Twenty-four milliliter of a CFDA/SE stock solution (50 mM in dimethyl sulfoxide) was added to the cells. The temperature of the reactor was cycled three times between 18°C and 35°C over the course of 2 h. This temperature cycling was found to result in the most optimum staining of cells during previous research [7]. Cells were harvested by centrifugation, washed twice with NCAGW, and resuspended in 12 l NCAGW. Cells were returned to the 20-l bioreactor and incubated at 18°C for approximately 24 h with stirring; the reactor window was covered with aluminum foil to minimize exposure of the cells to light. Prior to transport to the field, CFDA/SE-stained cells were harvested, washed once in NCAGW, and resuspended in 120 l NCAGW in a 470-l HDPE tank (McMaster-Carr Supply Company, Dayton, NJ, USA) at a nominal concentration of 1×10^9 cells ml^{-1}. The tank was covered with black plastic to protect the cell solution from exposure to sunlight.

While 90% of the DA001 cells prepared for injection were stained with CFDA/SE, 10% of the cells were isotopically labeled with ^{13}C, starting with a culture of ^{13}C-enriched cells. An initial 180-ml batch culture was grown using 0.2% (w/v) $^{13}CH_3^{13}COONa$ in BSM, which was then used to inoculate a 7-l autoclavable bioreactor (Applikon, Inc., Foster City, CA, USA); the reactor working volume was 5.2 l. Bioreactor parameters (temperature, pH, oxygen) were maintained as described above. ^{13}C-labeled sodium acetate was added over a 48-h period until the culture reached an OD_{550} of 2.7. ^{13}C-labeled cells were harvested by centrifugation, washed twice in NCAGW, and resuspended in 5 l NCAGW. Cells were incubated at 15°C for 36 h with shaking. Prior to transport to the field, cells were harvested, washed twice in NCAGW, re-

suspended in 5 l NCAGW, distributed to two 3.5-l carboys, and placed in a cooler with ice packs until injection in the field.

2.5. Initial bromide tracer injection

An injection of bromide was conducted on October 26, 1999 to assure that the hydraulics of the flow cell were operating properly. A 1230-l HDPE injection tank (McMaster-Carr Supply Company, Dayton, NJ, USA) was fitted with inlets for NC groundwater, bacteria and bromide additions, and an outlet for the mixed injection solution (located near the base of the tank). Another inlet was used to add a headspace of 3% CO_2 (v/v, in air) in air into the tank, which prevented the pH of the groundwater from increasing due to CO_2 off-gassing. Mixing of the tank was performed using a SandPiper PB1/4-A type 2 air-powered double-diaphram plastic pump (Model TT-2-PP, Warren-Rupp, Inc., Manfield, OH, USA), which withdrew liquid from the bottom of the tank and re-injected it into the top of the tank; the pumping rate was 4 l min^{-1}. Silicone sealant was applied around all fittings and the tank cover to make the vessel air tight. Three liters of NaBr solution (24 g Br l^{-1}) was mixed with approximately 720 l of NC groundwater to achieve a final bromide concentration of 100 mg Br l^{-1}. The bromide tracer solution was introduced at well B2 with a peristaltic pump at a rate of 1.0 l min^{-1}, using two inflatable straddle packers (Baski, Inc., Denver, CO, USA) to direct the solution into the aquifer at a depth of 7.8 to 8.3 m bgs. Additional formation water was introduced into well B2 above the injection zone at a rate of 4.0 l min^{-1}. The movement of the tracer through the MLS array was monitored by sampling the groundwater as described below.

Bromide concentrations were determined using a bromide electrode (Cole-Parmer, Vernon Hills, IL, USA) connected to a Corning pH/ion analyzer (Corning, Inc., Corning, NY, USA). Two liters of bromide standards (20, 50 and 100 mg Br l^{-1}) were prepared in NC groundwater at the start of the experiment. Individual 50-ml aliquots of the standards were used for three-point calibration of the probe on a daily basis. The high humidity and temperature variations at the NC site, especially at night, required that bromide measurements be performed in the RV. Samples and standards were amended with ionic strength adjuster (ISA, 5 M $NaNO_3$, 1.0 ml per 50-ml sample) and mixed on a stir plate during analysis.

2.6. Bacterial injection

The CFDA/SE-stained and ^{13}C-enriched DA001 cells were transported from Envirogen to the South Oyster site on October 28, 1999. A small compressor was used to keep the CFDA/SE-stained cell solution in the tank aerated, which was shown to help maintain cell viability during laboratory tests.

At the field site, the injection tank was placed under a small tent to shield it from direct sunlight. Both the CFDA/SE-stained and ^{13}C-labeled DA001 cells, and a concentrated solution of NaBr, were added to the 1230-l injection tank, brought to a final volume of 720 l with NC groundwater. The nominal concentrations of bacteria and tracer were 1×10^{11} CFDA/SE-stained DA001 cells l^{-1}, 1×10^{10} ^{13}C-labeled DA001 cells l^{-1}, and 100 mg Br l^{-1}, respectively. The injectate was mixed using the diaphram pump for the initial 5 h of the injection, but then the pump broke. The injectate was then mixed by rocking the tank every hour for the remainder of the injection period (9 h). Injection of the solution into well B2 (1.0 l min^{-1}) commenced at 17:35 on October 29, 1999 and lasted 12 h. A 'T' connector and valve were inserted into the tubing leading from the injection tank to well B2, which allowed samples from the injection tank to be collected approximately every hour during the injection. A peristaltic pump connected to tubing inserted into well B2 allowed the injection zone to be sampled during and after the injection. Samples from each of the batch of cells, and of the final mixture injected into B2, were transported back to Envirogen for enumeration of cells by plating and direct microscopic counts, and for determination of the percent adhesion of each batch of cells to native NC sediments using a standardized assay [14].

2.7. Groundwater sampling and processing

Sampling of groundwater for both the initial bromide injection and the bacterial injection was performed according to a predetermined schedule based on pre-injection modeling results [12]. Only ports P5 through P12 of each MLS were sampled because the injectate was not expected to migrate above P5 due to the depth of the injection zone. During the initial bromide injection, 50-ml samples from each port were collected at each timepoint and analyzed for tracer concentrations. During the bacterial injection, the initial 50 ml of groundwater from each port was assumed to represent a purge of the tubing and fittings, and was therefore collected and discarded. Two additional sets of 50-ml samples were collected into conical polypropylene centrifuge tubes and labeled with the MLS number (S1–S24), the Port number (P5–P12), the date and time. One set of samples was covered with black plastic and transported to the RV for processing. The other set of samples was placed on ice and shipped to the University of Montana for analysis using molecular and stable isotope methods [15]. Preliminary molecular and ^{13}C results from these analyses are published elsewhere [10] and will not be discussed further here.

An initial 1.5 ml of the Port 9 sample from all MLSs was poured into polypropylene Eppendorf tubes, labeled and placed at 4°C. The remainder of the sample was then preserved by adding 1.5 ml of 37% formaldehyde (w/v), resulting in a final preservative concentration of 1% (w/v).

Tubes were sealed and inverted several times to mix. Fifteen milliliters were split off into a 15-ml conical centrifuge tube, labeled, and placed on ice for shipment to the University of Utah for ferrographic capture analysis. After on-site microplate spectrofluorometry analysis, the remainder of each sample was placed on ice for shipment to Envirogen.

Periodically, 50-ml samples were also obtained from well M1 (using a peristaltic pump), and from wells M2, M3, M4 and B3 (using a sampling manifold) using tubing which was positioned to sample from approximately 8 to 8.5 m bgs. All samples were processed as described above.

2.8. Sample analyses

On-site enumeration (MP-Field) of CFDA/SE-stained cells was performed using a Molecular Devices SPECTRAmax GEMINI dual-scanning microplate spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA, USA) as described previously in Fuller et al. [7]. Ninety-six samples (0.35 ml) were pipetted into black OptiPlates (Packard Instrument Company, Meriden, CT, USA) and the fluorescence (Ex 495 nm, Em 538 nm, cutoff 530 nm) of each sample was measured. A dilution series was prepared using NC site groundwater and a composite of samples taken from the injection tank. Eight replicates of each dilution were pipetted in a microplate, and the fluorescence of each dilution was measured. A standard curve relating the stained cell concentration (based on direct counts (DCs) using epifluorescent microscopy (see below) of the stained cells prior to transport to the field) and fluorescence was used to convert sample fluorescence to CFDA/SE-stained cells ml^{-1}. The limit of detection in the field was determined to be 7×10^4 stained cells ml^{-1}.

Samples were analyzed in the laboratory for CFDA/SE-stained cells using microplate spectrofluorometry (MP-Laboratory). An early comparison indicated that MP-Field yielded different results than MP-Laboratory, which were found to be linked to the presence of non-cell-associated fluorescence in the samples due to loss of the fluorophore from the cells, or other autofluorescing compounds in the groundwater. To correct for this, eight replicates of each sample were analyzed using microplate spectrofluorometry before and after passage through a 0.2-μm nylon syringe filter. The average fluorescence of the eight filtered replicates was subtracted from the average of the eight unfiltered replicates, and this value was used to convert from sample fluorescence to cells ml^{-1} using a standard curve prepared in the laboratory from the most concentrated sample (based on direct epifluorescent microscopy) from MLS S2. The errors associated with the average fluorescence of filtered and unfiltered samples was propagated to calculate the error associated with the cells ml^{-1} value for each sample. The lower detection limits for MP-Laboratory, based on the point at which the filter-corrected fluorescence of the sample became greater than the filter-corrected fluorescence of the background, varied by well: S2, 5×10^4; S10, 2×10^5; S14, 1×10^5; S17, 3×10^5; S21, 2×10^5; S24, 2×10^5.

Enumeration of CFDA/SE-stained cells was also performed by DC using epifluorescence microscopy and FC as described in [7], and by ferrographic capture (FT) as described in Johnson et al. [10]. The effective detection limit for DC was 1×10^4, based on the largest volume of a sample that could be filtered onto the black polycarbonate membranes (10 ml) and requiring that at least 30 CFDA/SE-stained cells be counted on a given filter. The detection limits for FC and FT were determined by preparing serial dilutions of CFDA/SE-stained cells, and enumerating the cells in each dilution in triplicate [7,16]. Based on the cell concentrations at which analytical errors rose above 20%, effective detection limits for FC and FT were determined to be 1000 and 20 CFDA/SE-stained DA001 cells ml^{-1}, respectively. The bromide concentration in each sample was determined using an ion specific electrode as described above, with a detection limit of 0.5 mg l^{-1}.

2.9. Effects of sample storage and handling

FC data for the field samples was analyzed to determine the effects of storage temperature and storage duration on the CFDA/SE fluorescence per cell. Samples were amended with 1.0-μm carboxylate-modified TransFluorSpheres (TFS, Ex 488 nm/Em 645 nm, Molecular Probes, Inc., Eugene, OR, USA) prior to FC analysis. Both the geometric mean of cell fluorescence and median cell fluorescence were examined. The CFDA/SE-TFS ratio, defined as the ratio of DA001 cell fluorescence to TFS fluorescence, was calculated before comparisons were made to account for any variability in the flow cytometer optics and detection systems. The fluorescence of the TFS were assumed to be constant for a given set of FC parameters (i.e., photomultiplier tube voltage).

Additional laboratory experiments were conducted to examine the effects of light exposure during sample handling. A cell suspension of stained DA001 cells (approximately 5×10^6 cells ml^{-1}) was prepared in NCAGW and 40-ml volumes were distributed into sterile 50-ml polypropylene centrifuge tubes (Corning P/N 25330-50, Corning, Inc., Corning, NY, USA). One tube for each exposure treatment was wrapped completely with aluminum foil and served as the control. Additional tubes were treated as follows: exposure to overhead fluorescent lighting; exposure to a 60-W ($\sim$1300 lumen) incandescent light bulb at a distance of 15 cm; exposure to sunlight passing through window glass; exposure to direct sunlight. Aliquots from each tube were taken initially and then periodically for up to 18 h. Samples were analyzed by microplate spectrofluorometry to determine the effects of light expo-

sure on total fluorescence, and by FC to determine the effects of light on the distribution of fluorescence per cell and on cell concentrations.

3. Results and discussion

3.1. Effects of sample storage and handling

Exposure to direct sunlight caused a significant decrease in the total fluorescence of a suspension of CFDA/SE-stained cells (determined by MP-Laboratory), the per cell fluorescence, and the number of cells detected by FC (Fig. 2). Exposure to sunlight which had first passed though a pane of glass also reduced DA001 fluorescence, although to a lesser degree. Both of these experiments were performed in February 2000, and clouds partially blocked the sun during the latter portion of the exposure.

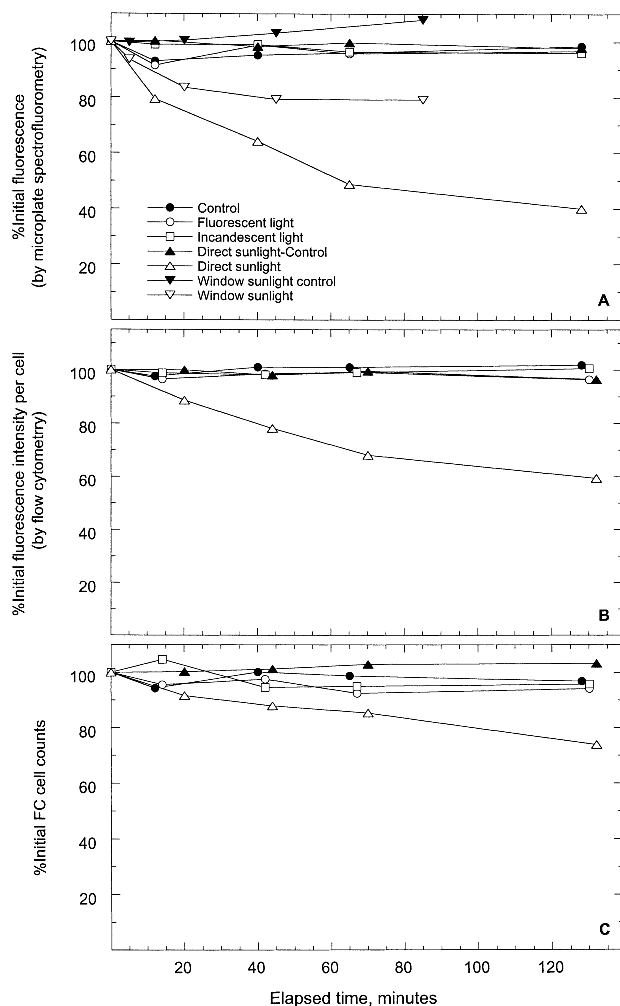

Fig. 2. Effects of exposure of solutions of CFDA/SE-stained DA001 cells to fluorescent light (○), incandescent light (□), direct sunlight (△), sunlight passed through window glass (▽), or no light (●, ▲, ▼). A: Percent of initial CFDA/SE fluorescence determined by MP-Laboratory. B: Percent of initial per cell fluorescence determined by FC. C: Percent of initial cell concentrations determined by FC.

Therefore, the effects of sunlight exposure were probably underestimated, and care should be taken to protect samples from exposure to sunlight, especially during the summer months under clear skies. Neither fluorescent nor incandescent lighting had a measurable effect on the sample fluorescence. Exposure of cell solution to short or long wavelength UV light (255 nm and 366 nm, respectively) also caused no fluorescence reductions (data not shown); this corroborates the effects caused by window-filtering of sunlight, which would not have allowed passage of the majority of the UV wavelengths. It is believed that the loss of fluorescence upon exposure to sunlight is due mainly to the intensity of the light, rather than the specific wavelengths of the light. Overall, the CFDA/SE-stained cells were relatively photostable under most conditions likely to be encountered during handling and analysis.

3.2. Monitoring of DA001 transport in the field

Horizontal planar contour plots based on results from Port 9 samples from throughout the MLS array at 24, 72 and 168 h are presented in Fig. 3. Data were graphed using triangulation with linear interpolation between discrete data points. This analysis revealed that the highest concentrations traveled along the centerline of the MLS array, with a slight drift to the western side further downgradient. The cell plume essentially tracked along with the bromide tracer center of mass. By the end of the forced gradient phase of the experiment, the highest cell concentrations were found in the vicinity of MLS S10.

This is believed to be the first report of near-real-time monitoring of bacterial transport under field conditions. Previous field-scale transport experiments have had to rely on laboratory analyses such as selective plating [17–20], direct microscopic counts [2–4,21,22], or observed biodegradation activity [23] to delineate the bacterial plume or cell transport distance. All of these are labor intensive, and results are not available for several days. The ability to monitor the extent of bacterial transport in real-time is crucial for effective bioaugmentation. For instance, an in situ technology for remediating chlorinated solvent-contaminated groundwater by injecting degradative organisms into the aquifer has been developed by Envirogen [24]. The technology requires cell densities of at least 10^6 cells ml^{-1} in the contaminated area (with injection concentrations of $\sim 10^{11}$ cells ml^{-1}) to achieve detectable contaminant degradation in a reasonable amount of time. Cell concentrations in the groundwater are normally determined using selective plate counts, with a 2–3-day lag time between sampling and results. In contrast, if the CFDA/SE staining were combined with MP-Field, the cell concentrations could be determined by field personnel in near-real time, allowing adjustments to made in the hydraulic gradient to facilitate even cell distribution, or injection of more cells as needed. Field samples could

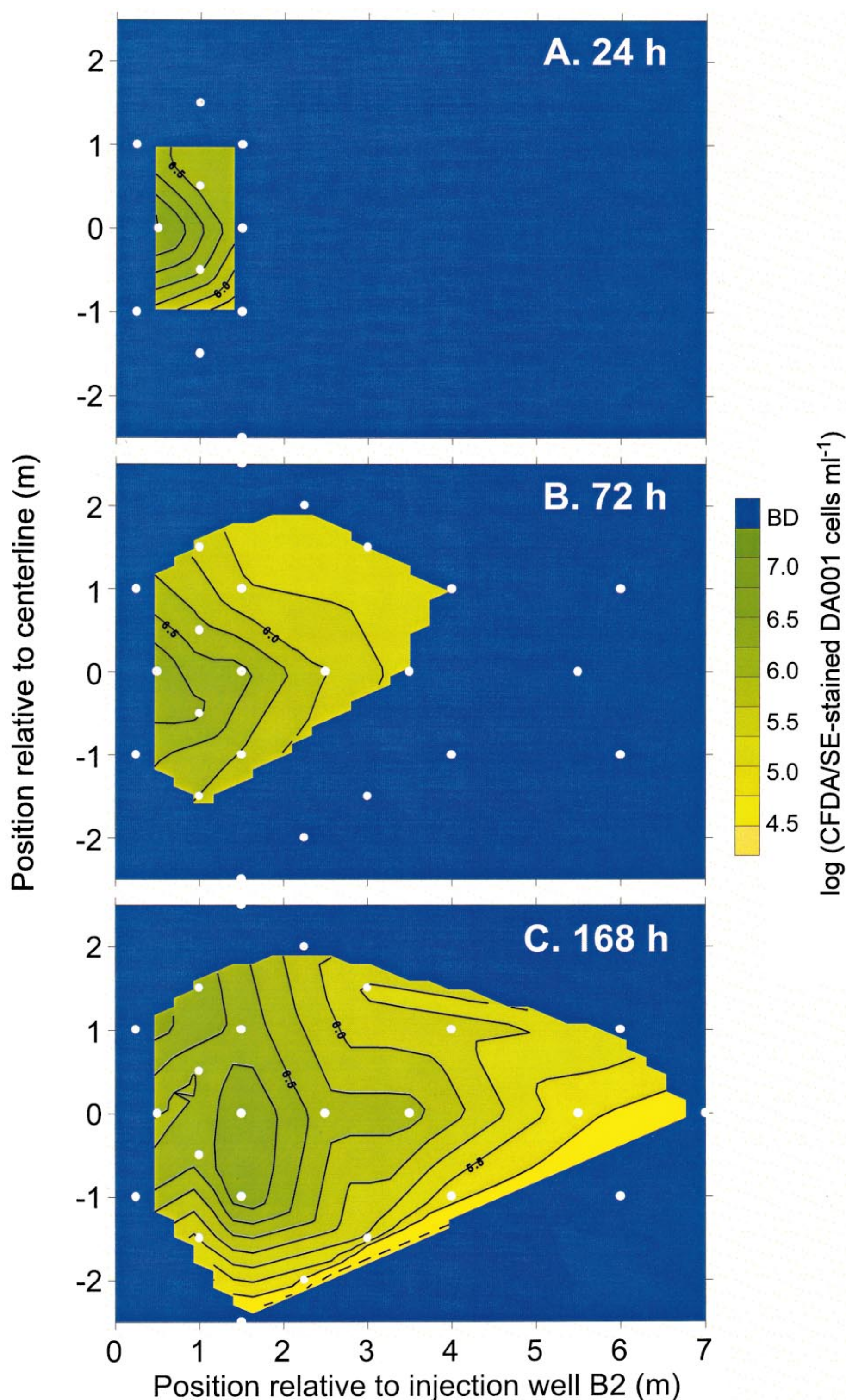

Fig. 3. Horizontal plane view of concentrations of CFDA/SE-stained DA001 cells in samples from Port 9 (8.17–8.33 m bgs) as determined by near-real-time MP-Field. A: 24 h post-injection. B: 72 h post-injection. C: 168 h post-injection. The MLSs being sampled at each timepoint are designated by the white circles. Concentrations at some MLSs were below detection, resulting in the MLS falling outside the contour lines defining the plume.

also be shipped to a laboratory for MP analysis, with a 1-day as opposed to a 2–3-day turnaround time. Although there would be some additional costs incurred for the CFDA/SE staining procedure, the ability to monitor the transport of the bacterial plume in near-real time should lead to cost-savings in the long-run. It must be noted, however, that if microplate spectrofluorometry is used in the field, the amount of background fluorescence should

be determined on selected samples using the filtering method used for the MP-Laboratory method described above to assess the extent to which the CFDA/SE stain is being released from the cells. Otherwise, an overestimation of the the number of cells in the samples may occur.

3.3. Comparison of detection methodologies

The results obtained using DC, MP (field and laboratory), FC and FT obtained from Port 9 of the centerline MLSs (S2, S10, S14, S17, S21 and S24) are presented in

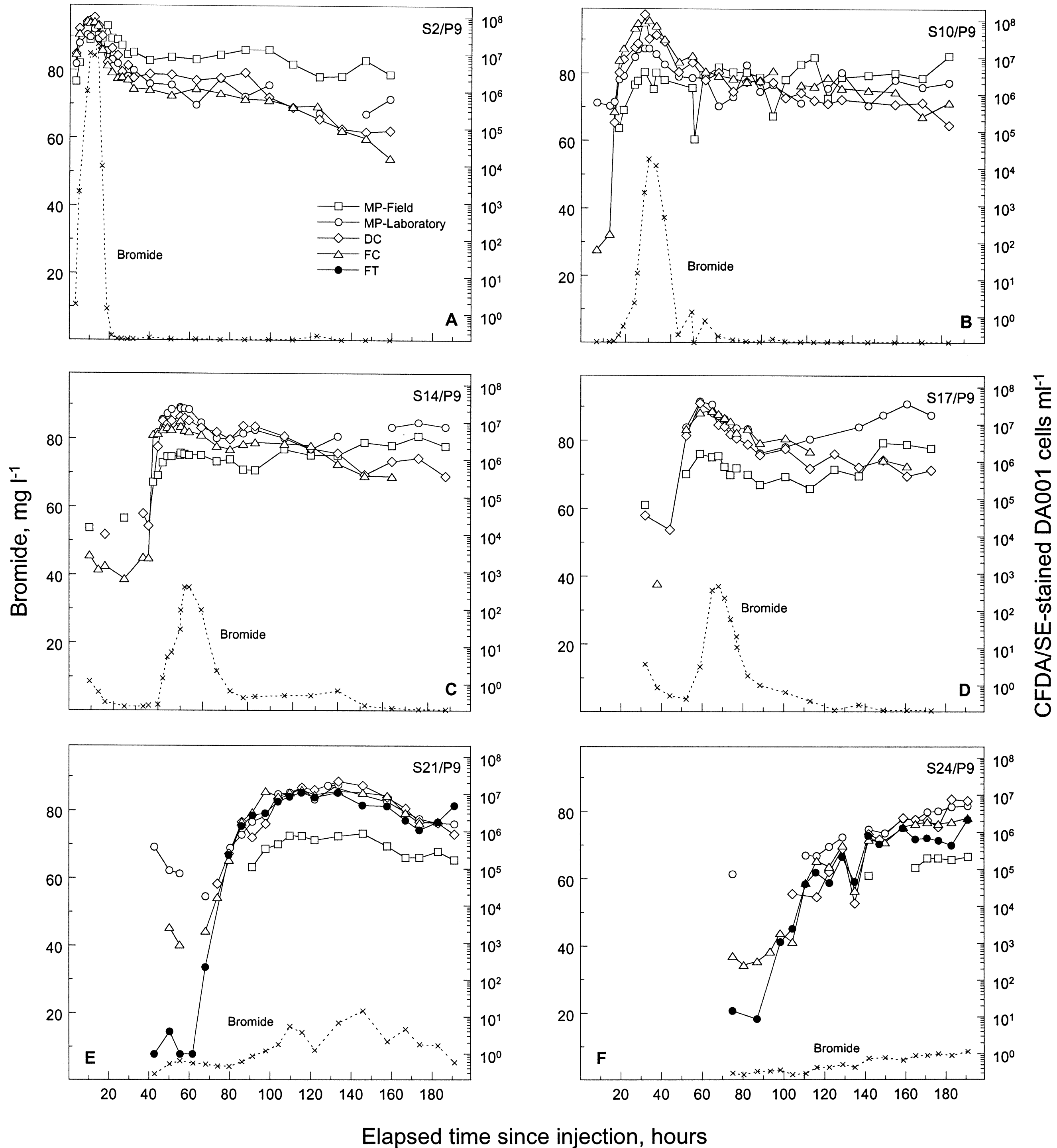

Fig. 4. Concentrations of CFDA/SE-stained DA001 cells in samples from Port 9 (8.17–8.33 m bgs) of the centerline MLSs based on MP-Field (□), MP-Laboratory (○), DC (◇), FC (△), and FT (●). Breakthrough of the conservative bromide tracer is also shown (-x-). Breaks in the curves indicate the presence of samples with concentrations that were below the detection limit for the method. A, S2; B, S10; C, S14; D; S17; E, S21; F, S24.

Fig. 4. Comparable cell concentrations were obtained using all the methods. Even with the somewhat greater disparity between the MP-Field results and the other methods, especially for samples from S2 and S21, the MP-field method was quite robust. The bacterial peak arrived at sequentially later times along the centerline. The peak concentration of CFDA/SE-stained DA001 cells at each MLS remained quite high, and the ratio of the concentration in the sample to the concentration injected (C/C_0) was still 0.1 at S24, 7 m downgradient from the injection well.

Results based on MP-Field, MP-Laboratory, FC and FT, from Ports 5–12 of MLS 21 are presented in Fig. 5. Samples from P5 were not analyzed using MP-Laboratory or FC. MP-Field and MP-Laboratory analyses detected bacterial breakthrough in P9, P10 and P12; there were some spurious 'hits' of stained cells in P5, P7, P8, and P11 during MP-Field measurements. FC and FT revealed that CFDA/SE-stained DA001 cells arrived sooner, and were more vertically distributed than either MP-Field or MP-Laboratory analysis would indicate due to the lower detection limits for the FC and FT methods. Where there is data from all four methods, the results are in agreement, with the exception that the MP-Field analyses yielded approximately 10-fold lower concentrations of cells than the other three methods. Concentrations at or around 10^7 stained cells ml^{-1}, which represents a C/C_0 of 0.1, were seen in P8, P9, P10, and P12; concentrations in P11 reached a maximum of 10^5 cells ml^{-1}. Given that the vertical distance between P8 and P12 of S21 was approximately 1.1 m (center-to-center), and the cells were injected over a 0.5-m vertical interval in B2, the combined results using all four detection methods indicate that the plume

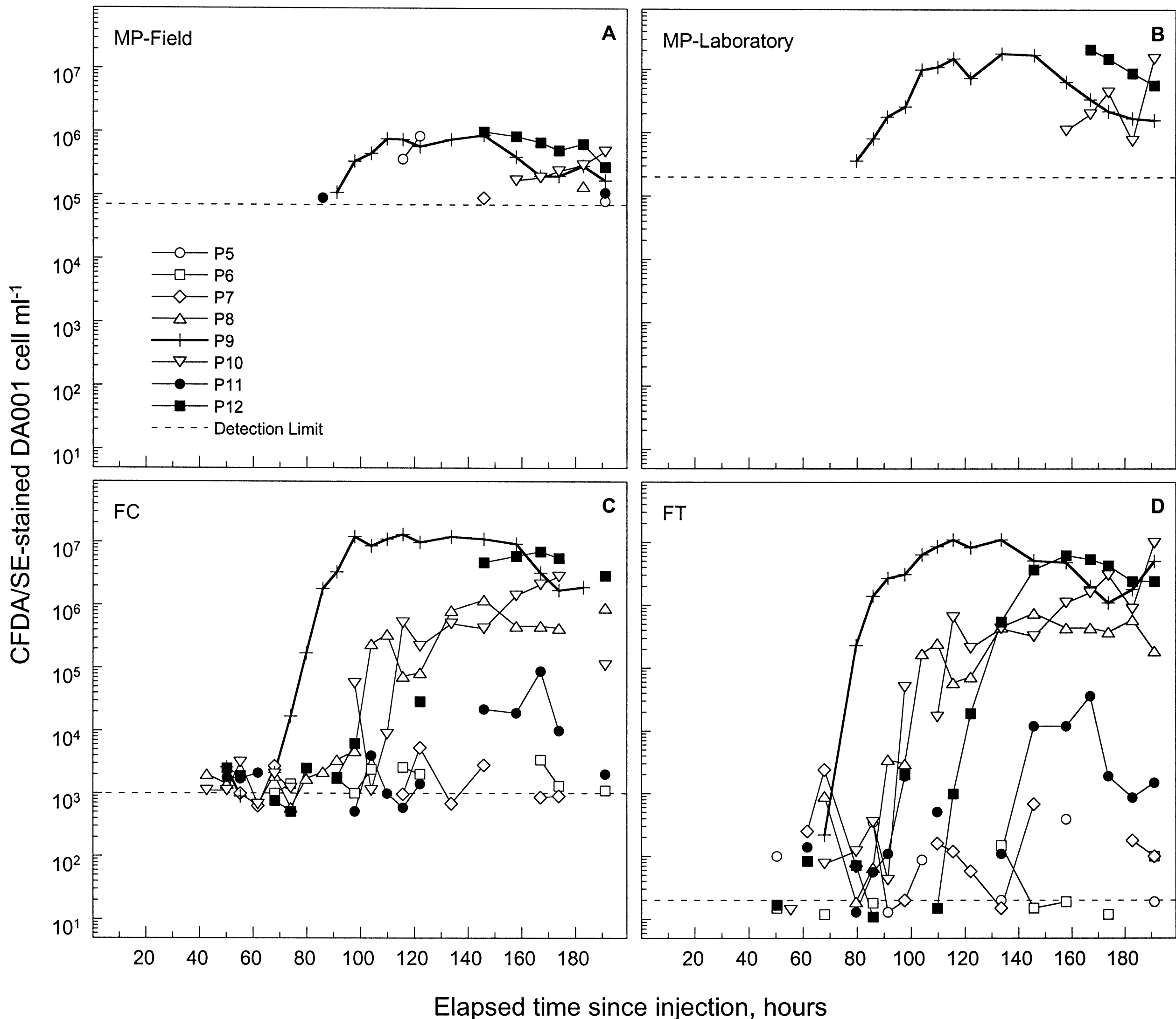

Fig. 5. Concentrations of CFDA/SE-stained DA001 cells in samples from Port 5 to Port 12 of S21. Breaks in the curves indicate the presence of samples with concentrations that were below the detection limit for the method. The dashed horizontal line represents the lower detection limit of each method. A, MP-Field; B, MP-Laboratory; C, FC; D, FT.

remained relatively tight, moving vertically only 0.1 m for every 1 m of horizontal movement. The results also indicate a slight downward movement of the bacterial plume, and preferential flow just above the aquifer confining layer, especially further downgradient from the injection well.

FC analysis of samples indicated that the CFDA/SE fluorescence per DA001 cell was not significantly different in samples taken close to or far from the injection point, or in samples taken early versus late in the experiment. For instance, samples taken from S2 and S24 had identical CFDA/SE-TFS ratios (based on the median particle fluorescence values) of 0.14 ± 0.01; samples taken from S2 and 3 h and 159 h had CFDA/SE-TFS ratio values of 0.14 ± 0.01 and 0.13 ± 0.01, respectively. Ratios calculated using the geometric mean of fluorescence were essentially identical to those calculated using the median fluorescence (data not shown). Cells in samples taken over the 5 months after injection had similar CFDA/SE-TFS ratios as samples taken within the first week after injection. The fact that the per cell fluorescence did not decrease indicates that the at least some of the injected cells never underwent cell division, which would have resulted in much lower CFDA/SE-TFS ratios.

Detectable concentrations of CFDA/SE-stained DA001 cells were present in Port 9 of selected centerline MLSs well after the forced gradient phase of the experiment was completed (Fig. 6). There was significant disagreement between the results obtained with the MP-Laboratory, FC and FT methods for S2, whereas FC and FT yielded comparable trends of decreasing cells for S14 and S21 over the 5-month period. An increased level of natural, autofluorescing material in the groundwater may be one reason why MP-Laboratory detected higher apparent concentrations of cells than either FC or FT, since MP measures the total fluorescence of a sample, regardless of whether the fluorescence is associated with the introduced CFDA/SE-stained cells or not. The fluorescence of a 0.2-μm-filtered aliquot of each sample was measured and subtracted from the non-filtered sample fluorescence, but this corrected fluorescence was still quite high – i.e., the S2/P9 sample taken 150 days after injection had a total fluorescence of approximately 14 relative fluorescence units (RFU), which translates to cell density of 3×10^6. However, this RFU to cells ml^{-1} conversion is based on the standard curve developed at the beginning of the experiment, and may not be applicable for samples collected over the entire 5-month period. For the latest samples taken, FC analysis gave results which were one order of magnitude higher than FT. The FC analysis also indicated that the per cell fluorescence increased slightly over this period of time. This may reflect (1) the ability of the flow cytometer to detect and count stained cells which are not visible to the human eye under the microscope during FT analysis; (2) the inclusion of (auto)fluorescent particles that are not cells in the FC count, or; (3) decreases in the capture efficiency of

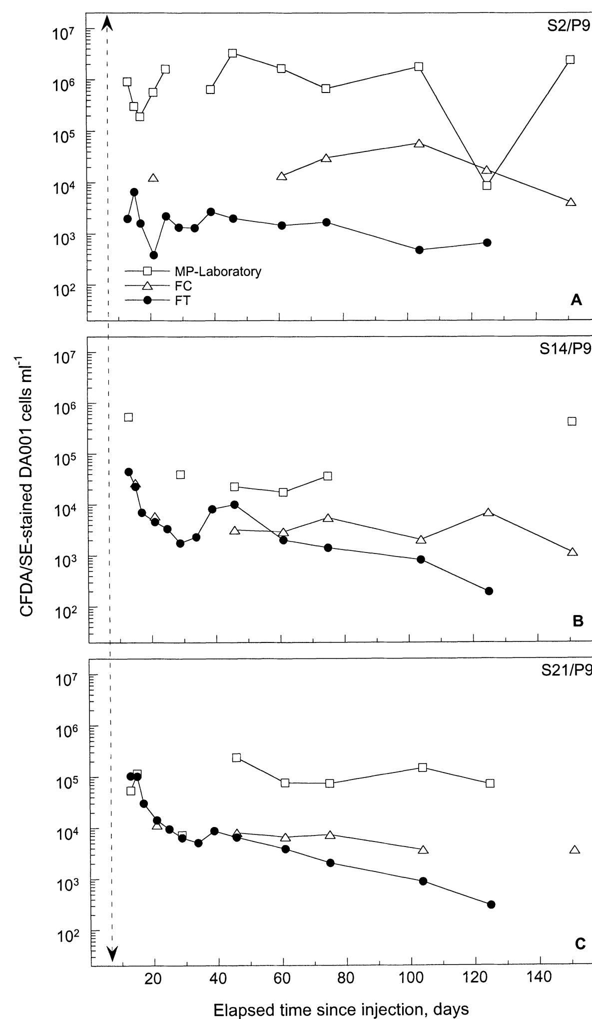

Fig. 6. Concentrations of CFDA/SE-stained DA001 cells in selected samples collected after the forced hydraulic gradient was terminated, as determined by MP-Laboratory (□), FC (△), and FT (●). All analyses were performed in the laboratory. Breaks in the curves indicate the presence of samples with concentrations that were below the detection limit for the method. A, S2/P9; B, S14/P9; C, S21/P9.

FT for the target cells due to changes in the cell surface properties over time. Fuller et al. [7] determined that the error associated with FC analysis of CFDA/SE-stained DA001 only increased significantly below 500 cells ml^{-1}, but there are differences between FC and FT in Fig. 6 starting at 10^4 cells ml^{-1}. Discrepancies between FC and FT were observed by Johnson et al. [10] for sample concentrations between 10 and 1000 cells ml^{-1}, with FC yielding higher cell counts than FT. Since the discrepancies in Johnson et al. [10] were for samples collected only a short time (< 1 week) after injection, the differences were not likely due to changes in the cell surface causing reduced FT capture efficiency. Additional experiments have not indicated any significant change in FT capture efficiency in unpreserved samples over a period of up to 30 days (Dr. W.P. Johnson, University of Utah, UT, USA, personal

communication). Taken together, it seems likely that the differences between FC and FT observed in Fig. 6 for samples collected from 50 days post-injection onward was due to some bias in FC analysis (i.e., counting particles that are not cells). More specific experiments will be needed to verify this.

These analyses revealed the excellent longevity of the CFDA/SE staining in non-growing cells, even under the likely stressful conditions encountered by the DA001 cells in situ. This indicates that this stain, coupled with the various detection methods, would be very suitable for determining the residence time of degradative organisms after being introduced into a contaminated aquifer.

The three main methods examined during this work – MP, FC, and FT – had different lower detection limits (10^5, 1000, 20 cells ml^{-1}, respectively). There were also differences in the analysis time per sample (1, 2 and 12 min, respectively) and the cost per sample ($0.25, $1.31, and $1.87, respectively, excluding labor). However, looking at all the MP-Field, FC, and FT data indicated that a tiered approach to sample analysis might be applied to reduce both the total time and costs associated with processing large numbers of samples, while still achieving excellent resolution. All samples would initially be screened using the MP method, either in the field or in the laboratory. FC analysis would then be performed on any sample with a concentration below the MP detection limit, followed by FT analysis for any sample below the FC detection limit. It is likely that MP analysis would suffice for most bioaugmentation monitoring, since approximate versus exact cell concentrations would be sufficient to assess the progressive transport of injected cells within an aquifer.

In conclusion, this research demonstrated the feasibility of using fluorescently labeled bacteria and several enumeration methods with different detection limits to monitor the transport of introduced bacteria in the subsurface. Efforts to monitor a co-injection of two different bacterial strains, each stained with a different color fluorophore, are currently being planned, as well as refinements to the MP-Field protocol to achieve more accurate results.

Acknowledgements

This research was funded by the NABIR Program program, Biological and Environmental Research (BER), U.S. Department of Energy (Grant No. DE-FG02-98ER62712). The authors would like to acknowledge the leadership of Program Manager Dr. Frank Wobber. We would also like to thank Mr. Tim Griffin, Golder Associates for his excellent management of the field site operations. Access to the field site was granted by The Nature Conservancy. The assistance of research interns Ms. Katherine Tugusheva, Ms. Jessica Seebald, and Mr. Kevin Long was much appreciated.

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