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Application of a Vital Fluorescent Staining Method for Simultaneous, Near-Real-Time Concentration Monitoring of Two Bacterial Strains in an Atlantic Coastal Plain Aquifer in Oyster, Virginia

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Two differentially labeled bacterial strains were monitored in near-real time during two field-scale bacterial transport experiments in a shallow aquifer in July 2000 and July 2001. *Comamonas* sp. strain DA001 and *Acidovorax* sp. strain OY-107 were grown and labeled with the vital fluorescent stain TAMRA/SE (5 [and -6]-carboxytetramethylrhodamine, succinimidyl ester) or CFDA/SE (5 [and -6]-carboxyfluorescein diacetate, succinimidyl ester). Fluorescently labeled cells and a conservative bromide tracer were introduced into a suboxic superficial aquifer, followed by groundwater collection from down-gradient multilevel samplers. Cells were enumerated in the field by microplate spectrofluorometry, with confirmatory analyses for selected samples done in the laboratory by epifluorescence microscopy, flow cytometry, and ferrographic capture. There was general agreement in the results from all of the vital-stain-based enumeration methods, with differences ranging from <10% up to 40% for the analysis of identical samples between different tracking methods. Field analysis by microplate spectrofluorometry was robust and efficient, allowing thousands of samples to be analyzed in quadruplicate for both of the injected strains. The near-real-time data acquisition allowed adjustments to the predetermined sampling schedule to be made. The microplate spectrofluorometry data sets for the July 2000 and July 2001 experiments allowed the transport of the injected cells to be related to the site hydrogeology and injection conditions and enabled the assessment of differences in the transport of the two strains. This near-real-time method should prove effective for a number of microbial ecology applications.

The application of bioaugmentation to facilitate the remediation of soil and groundwater contaminated with recalcitrant pollutants has proven effective for several organic compounds (3, 15, 17, 18) and is expected to become more prevalent for the remediation of metals and radionuclides (6). One requirement for successful bioaugmentation is that effective concentrations of the injected microorganisms be distributed throughout contaminated areas of the subsurface.

The development of robust methods to track viable cells through the subsurface has proved to be quite challenging. Traditional staining methods (i.e., acridine orange and 4′,6′-diamidino-2-phenylindole [DAPI]) have been hampered by the effects of the stains on cell viability and cell surface properties. Nucleic acid-based techniques have previously been limited by high detection limits and/or interference from indigenous organisms. Genetic engineering can be utilized to enhance cell tracking (i.e., by insertion of green fluorescent protein markers), but regulatory concerns about the release of genetically modified organisms currently make these methods impractical. Recent studies have started to make progress on this topic by utilizing multiple tracking techniques to follow one species of bacteria through an aquifer for up to 1 year (9; unpublished data). For complex remediation projects and progress in the understanding of microbial movement through the subsurface to occur, it will be necessary to concurrently track multiple species of bacteria through the subsurface accurately and reproducibly.

Two field experiments were conducted in consecutive years (2000 and 2001) for which two strains of bacteria were labeled with vital fluorescent stains, coinjected, and tracked in a shallow coastal aquifer. The results presented here focus on field validation of the vital fluorescent staining and multiple cell tracking techniques. The results reported here relate specifically to the ability to simultaneously track both bacterial strains through the subsurface in near-real time and to show that microbial properties were the main determinant in the control of transport.

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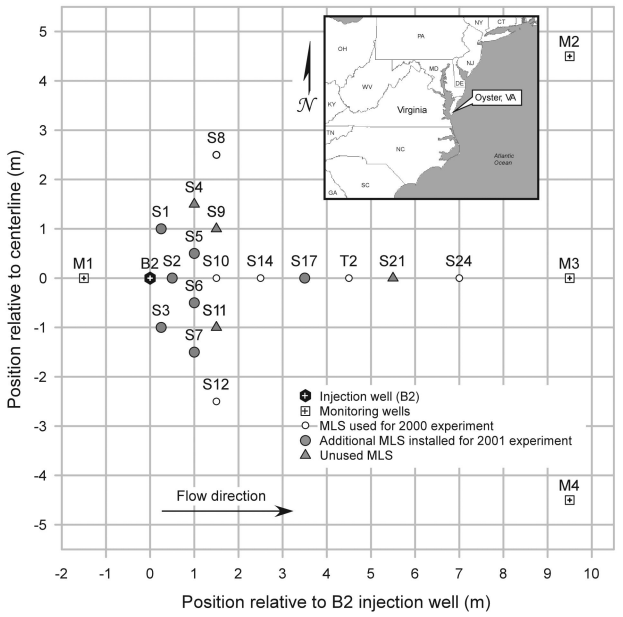

FIG. 1. Layout of the SOFA during the July 2000 and July 2001 field experiments. Bacteria and bromide were injected at well B2. The different symbols indicate the locations of the MLSs, which varied slightly for the two field experiments. The inset is a map showing the eastern seaboard of the United States and the location of Oyster, Va. (The map was adapted from one obtained from the University of Texas at Austin.)

MATERIALS AND METHODS

Site description. The experiments were conducted at the South Oyster Focus Area (SOFA) of the U.S. Department of Energy Natural and Accelerated Bioremediation Research (NABIR) Program South Oyster bacterial transport field site. The SOFA site is situated in shallow Atlantic Coastal Plain sediments within the Columbia aquifer on the eastern shore of Virginia (37°17′11″N, 075°55′22″W) (Fig. 1, inset). The bacterial transport experiments were performed with a multilevel sampler (MLS) array within the SOFA flow cell (Fig. 1). The longitudinal axis of the MLS array was aligned parallel to the natural groundwater flow direction, and a mean flow velocity of 1.0 m/day was established through the transport area. The hydraulic head across the flow cell did not vary by >0.02 m perpendicular to the direction of groundwater flow throughout the experiment. There were slight differences in numbers and locations of MLSs during the July 2000 and July 2001 experiments, as indicated in Fig. 1.

The portion of the aquifer in which these experiments were conducted is below a buried peat layer, and the sediments are predominantly fine- to medium-grain sands. The variations in saturated hydraulic conductivity represented by these different sedimentary materials at the SOFA flow cell were estimated by crossborehole tomography (Fig. 2). In Fig. 2, areas of high permeability are represented in blue and green, while areas of low permeability are represented in red and orange. The groundwater is anoxic (<1 mg of O_2/liter), contains low levels of dissolved and total organic carbon (<1 mg/liter), and has a subneutral pH (5.5 to 6.0 standard units). More complete geological descriptions of the site can be found in the work of Hubbard et al. and Chen et al. (1, 8). Each MLS had 12 ports spaced from −2.8 to −6.2 m below mean sea level (msl) (4.5 to 7.8 m below the ground surface) at approximate intervals of 0.3 m (white and black circles in Fig. 2), and samples were taken with a specially designed manifold (14). The top port was located below a low-permeability peat zone, ports 2 to 8 were located in a higher permeability zone, and the bottom ports were located in a lower permeability zone.

Chemicals and media. CFDA/SE (5 [and -6]-carboxyfluorescein diacetate, succinimidyl ester) was purchased from Fluka Chemical Company (Ronkonkoma, N.Y.). TAMRA/SE (5 [and -6]-carboxytetramethylrhodamine, succinimidyl ester), *N*,*N*-dimethylformamide, and anhydrous dimethyl sulfoxide were purchased from Sigma Chemical Company (St. Louis, Mo.). The two vital fluorescent stains were shown in previous research to have no adverse effects on cell viability while being retained in cells for long periods of time (5). Artificial groundwater (SOAGW, pH 6.0) was based on the SOFA site groundwater chemistry and was composed of the following (in milligrams per liter of double-distilled water): $MgSO_4 \cdot 7H_2O$, 100; KNO_3, 11; $NaHCO_3$, 120; $CaCl_2 \cdot 2H_2O$, 40; $Ca(NO_3)_2 \cdot 4H_2O$, 5; $CaSO_4 \cdot 2H_2O$; and NaH_2PO_4, 0.2. Phosphate-buffered saline (PBS; pH 7.4) was described previously (4).

Bacterial strains. Two adhesion-deficient variants of indigenous bacterial isolates were used for these field experiments. Adhesion-deficient strains were selected, as they represent one approach toward overcoming problems with dispersing degradative bacteria throughout contaminated aquifers in order to promote bioaugmentation (18). The first strain, DA001, was isolated from groundwater from the oxic aquifer underlying the Narrow Channel focus area of the NABIR South Oyster bacterial transport field site, approximately 0.75 km from the location of this experiment. The organism is a nonmotile, gram-negative, aerobic rod (1.2 μm long by 0.6 μm in diameter) identified as a *Comamonas* sp. (5). The second strain, OY-107, was isolated from a subsurface sediment from SOFA in the bacterial transport field site. Further characterization indicated that OY-107 was a nonmotile, gram-negative, facultative iron-reducing rod (1.9 μm long by 1.0 μm in diameter), tentatively identified by using 16S rRNA gene sequence data as an *Acidovorax* sp. (D. Balkwill, Florida State University, personal communication). These strains were selected for use during the field experiments because they met the criteria established by both the owner of the field site (The Nature Conservancy) and the overall project goals: (i) they were indigenous to the aquifer into which they were being injected, (ii) they were not resistant to any antibiotics of clinical significance, (iii) they were not known human pathogens, and (iv) they were adhesion deficient. Routine growth of the organisms was performed with 0.2% (wt/vol) sodium acetate and 0.1% (wt/vol) lactate in basal salts medium (7) at room temperature. Plating was performed on R2A agar (Fisher Scientific, Fair Lawn, N.J.).

Bacterial growth and labeling. Large volumes of *Comamonas* sp. DA001 and *Acidovorax* sp. OY-107 cells for both field-scale injections were grown aerobically on sodium lactate. Staining was performed when the DA001 and OY-107 biomasses reached cell densities of approximately 10^{10} cells/ml. Prior to staining, cultures of *Comamonas* sp. DA001 and *Acidovorax* sp. OY-107 cells were harvested in a CEPA Z41 flowthrough centrifuge (Carl Padberg Zentrigugenbau GmbH, Geroldsecker Vorstadt, Germany) operating at 17,000 × *g* and maintained at 4°C. Cells were washed twice in PBS and resuspended at a cell density of approximately 10^{10} cells/ml in a final volume of 10 liters of PBS. For the July 2000 injection, DA001 cells were stained with TAMRA/SE at a final concentration of approximately 120 μM (12.7 ml of a 100 mM stock in *N*,*N*-dimethylformamide) and OY-107 cells were stained with CFDA/SE at a final concentration of 120 μM (25 ml of a 50 mM stock in dimethyl sulfoxide). For the July 2001 injection, DA001 was stained with CFDA/SE and OY-107 was stained with TAMRA/SE to allow cells from the July 2001 injection to be distinguished from residual cells from the July 2000 injection. The staining procedure was performed in a BL 20.2-liter fermentor (LSL Biolafitte, Inc., Minneapolis, Minn.), with the temperature of the reactor cycling five times between 25 and 39°C. Cells were harvested by centrifugation when the staining procedure was completed, washed twice with SOAGW, and resuspended in 10 liters of SOAGW. Cells were transferred to 20-liter plastic carboys and incubated at 15°C with gentle shaking; the cells were aerated with an aquarium air pump. After 48 h, stained cells were again harvested by centrifugation and were washed with SOAGW until no detectable fluorescence remained in the supernatant, as determined by microplate spectrofluorometry. Washed DA001 and OY-107 cells in anoxic SOAGW were transferred to individual sealed 60-liter high-density polyethylene tanks (McMaster-Carr Supply Company, Dayton, N.J.), and the volume of each tank was brought to 45 liters with sterile anoxic SOAGW, resulting in an approximate density of 2×10^9 stained cells/ml. Each tank was wrapped in two black plastic trash bags to protect the cell solutions from light. The tanks were then placed in large plastic barrels and stored at 15°C until transport to the field site.

Bromide tracer solution preparation. The bromide (Br) tracer solution was prepared by dissolving 232 g of NaBr in 2 liters of SOAGW. This solution was added to a 60-liter high-density polyethylene tank, and the volume was brought to 45 liters with sterile, anoxic SOAGW, resulting in a Br solution with a final concentration of 4,000 mg/liter.

Cell and Br tracer injection. The year 2000 injection occurred from 31 July to 1 August, and samples were collected from the MLSs until 11 August, at which point the experiment was terminated. The year 2001 injection was performed from 16 July to 17 July, and samples were collected from the MLSs until 24 July. Anoxic groundwater for preparing the injection solution was obtained from a source well located 30 m south of injection well B2. A complete and detailed description of the injection system is presented elsewhere (14). The basic com-

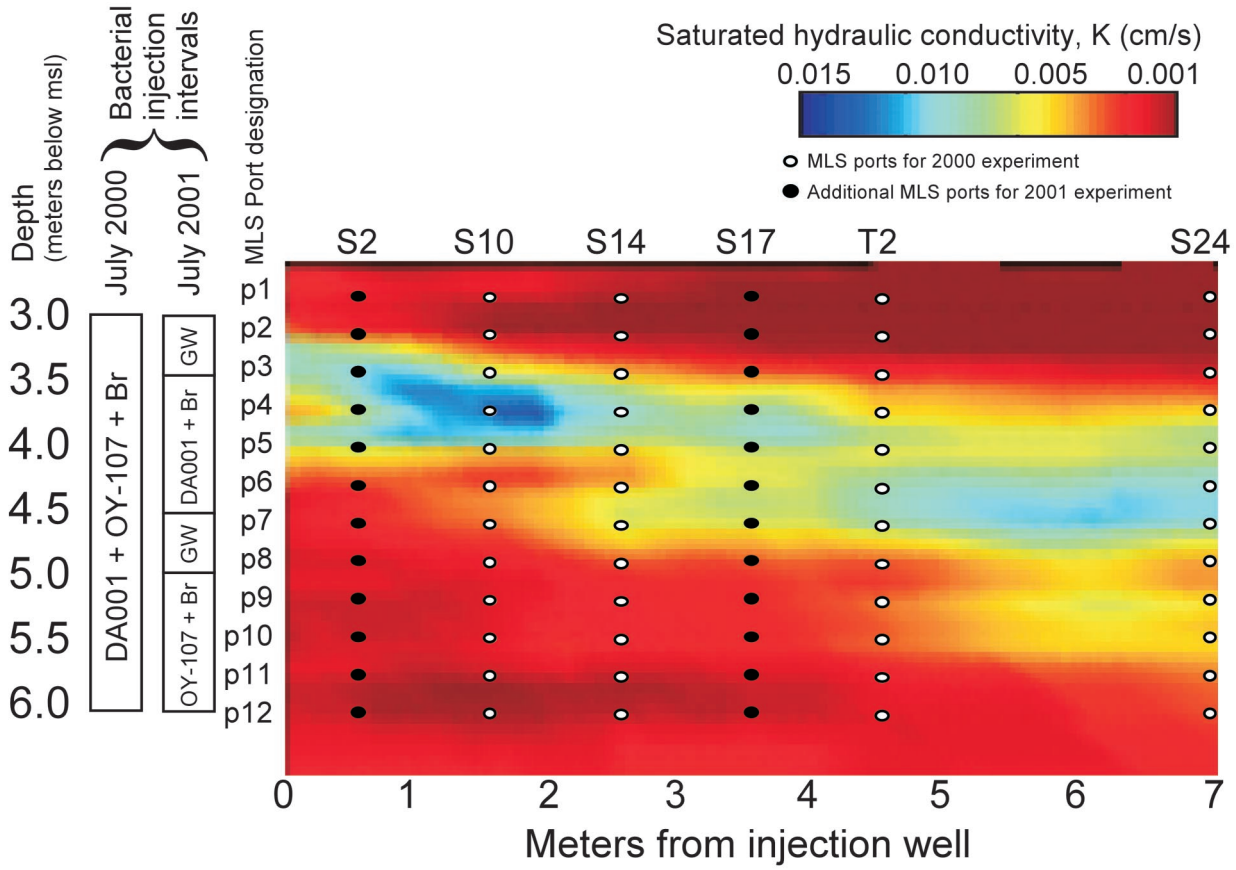

FIG. 2. Positions of the bacterial injection zones and the centerline MLSs and ports relative to the site hydrogeology for the July 2000 and July 2001 field experiments. Blue and green indicate areas of high permeability within the aquifer, while red and orange indicate low-permeability areas. The injection intervals for the bacteria and bromide are indicated by the vertical rectangles along the left side of the figure. The locations of the MLSs and the ports of each MLS are indicated by black and white circles. The depth is given as meters below mean sea level. GW, groundwater.

ponents were the three sealed tanks containing the concentrated *Comamonas* sp. strain DA001, *Acidovorax* sp. strain OY-107, and Br solutions; the injection solution mixing tanks; and the pumps, tubing, and metering valves used to combine, mix, and introduce the injection solution into well B2 of the SOFA flow cell. This mixing resulted in calculated concentrations of both DA001 and OY-107 of 5×10^7 cells/ml and of 100 mg of Br/liter in the final injection solution.

The injection zones for the two field experiments are depicted as vertical rectangles on the left side of Fig. 2. During the July 2000 injection, *Comamonas* sp. strain DA001, *Acidovorax* sp. strain OY-107, and Br were mixed together and injected into a 3-m interval within well B2 (−3.0 m to −6.0 m below msl). For the July 2001 field experiment, strains DA001 and OY-107 were combined with Br in separate tanks and injected into B2 at intervals of −3.5 to −4.5 m msl and −5.0 to −6.0 m msl, respectively. Two 0.5-m intervals, one directly above the DA001 injection zone and one between the DA001 and OY-107 injection zones, received only groundwater from the source well. The total combined flow into well B2 was 2.5 liters/min. Samples of the injection solution were collected during both experiments every 30 min for the analysis of cells and Br. When all of the injection solution had been introduced into well B2, the flow of the anoxic groundwater from the source well was switched to bypass the mixing tank and to go directly into well B2 at a rate of 2.5 liters/min for the duration of the experiments.

Groundwater sampling and processing. The sampling of groundwater was performed according to a predetermined schedule based on preinjection modeling results (16). Fifty-milliliter samples were collected in clean polypropylene centrifuge tubes. All samples were labeled, placed in coolers on ice, and transported to the field house for processing. Sample splits were removed and shipped to various laboratories for enumeration of the injected *Comamonas* sp. strain DA001 and *Acidovorax* sp. strain OY-107 cells by quantitative PCR (12), flow cytometry, ferrographic capture, plate counts, and epifluorescence microscopy direct counts (see below). The remainder of the sample was analyzed in the field by microplate spectrofluorometry.

Sample analyses. On-site enumeration of TAMRA/SE-stained DA001 cells and CFDA/SE-stained OY-107 cells was performed by microplate spectrofluorometry as described by Fuller et al. (5), with slight modifications. Four replicate 0.35-ml aliquots of each groundwater sample were analyzed in 96-well black OptiPlates (Packard Instrument Company, Meriden, Conn.) in a Molecular Devices SPECTRAmax GEMINI dual-scanning microplate spectrofluorometer (Molecular Devices Corporation, Sunnyvale, Calif.). The fluorescence in each well was measured twice, first for CFDA/SE fluorescence (excitation [Ex], 495 nm; emission [Em], 538 nm; cutoff, 530 nm) and then for TAMRA/SE fluorescence (Ex, 560 nm; Em, 585 nm; cutoff, 570 nm). A dilution series was prepared from the concentrated DA001 and OY-107 cell solutions and also from samples taken from the injection tank, and the total fluorescence of each dilution at the two Ex-Em wavelength combinations was measured. Samples of site groundwater were included in the series to account for background fluorescence. Standard curves relating the total cell concentrations (based on direct counts by epifluorescence microscopy) and total fluorescence at the two different Ex-Em wavelength combinations were used to convert the fluorescence of each sample to numbers of DA001 and OY-107 cells per milliliter of sample. Both viable and nonviable stained cells were included in the enumeration. A correction was also applied to account for the slight amount of TAMRA/SE fluorescence produced during the measurement of CFDA/SE fluorescence. The lower limits of detection for strains DA001 and OY-107 during the 2000 field experiment were 9.3×10^4 and 1.3×10^5 stained cells/ml, respectively. During the 2001 field experiment, the detection limits for DA001 and OY-107 were 6.3×10^4 and 3.2×10^5 stained cells/ml, respectively. During these field experiments, the average error associated with the analysis of four replicates of the same sample by microplate spectrofluorometry was 15 ± 6%.

The enumeration of CFDA/SE-stained cells was performed in the laboratory by epifluorescence microscopy direct counts (simply called "direct counts" henceforth) and flow cytometry as described by Fuller et al. (5) and by ferrographic capture as described by Johnson et al. (10, 11). Single replicates of field samples were analyzed by direct counting, ferrographic capture, flow cytometry, and spread plating. Based on previous research, the effective detection limits and standard deviations for replicate measurements of the same sample were as follows: direct counts, $1.0 \times 10^4 \pm 0.2 \times 10^4$ cells/ml; ferrographic capture, 20 ± 2 cells/ml; flow cytometry, $1{,}000 \pm 80$ cells/ml (CFDA/SE-stained cells only); and spread plating, 100 ± 10 culturable heterotrophic CFU per ml (2).

Flow cytometry for TAMRA/SE-stained DA001 or OY-107 cells was deemed to be unreliable due to the incompatibility between TAMRA/SE fluorescence Ex and Em wavelengths and the light source, filters, and detector employed by the flow cytometer. However, an attempt was made to gate the raw flow cytometry data so that CFDA/SE-stained cells could be subtracted from total cells to arrive at counts of TAMRA/SE-stained cells.

Br concentrations were determined both on-site and in the laboratory by using a bromide electrode (Cole-Parmer, Vernon Hills, Ill.) connected to a pH-ion analyzer (Corning, Inc., Corning, N.Y).

RESULTS AND DISCUSSION

Cell staining with CFDA/SE versus TAMRA/SE. Both CFDA/SE and TAMRA/SE were highly efficient at staining cells. TAMRA/SE-stained cells often had a stronger fluorescence than CFDA/SE-stained cells by epifluorescence microscopy and hence may be easier to detect and count under some circumstances (i.e., in the presence of large amounts of autofluorescing material such as humic material or sediment). The detection methods that will be used to enumerate labeled cells and the properties of the sample matrix need to be taken into consideration when deciding which of the two vital stains will be most effective in a given situation.

These were the first large-scale preparations of TAMRA/SE-stained cells. The costs for bulk quantities of CFDA/SE and TAMRA/SE were similar, but for the quantities required for these experiments, the availability of TAMRA/SE was more limited than CFDA/SE. Compared to the large-scale preparation of CFDA/SE-labeled cells, the preparation of cells labeled with TAMRA/SE required more washing steps to remove excess non-cell-associated stain. These translated to a greater expenditure of time and materials when preparing the TAMRA/SE- versus CFDA/SE-stained cells.

Comparison of detection methodologies for *Comamonas* sp. DA001 and *Acidovorax* sp. OY-107. The results obtained with the different enumeration methods were comparable to each other (Figs. 3 and 4). The shapes of the breakthrough curves for the two organisms at a given location in the field were parallel for each of the methods used. The average correlation coefficients (r) for pairwise comparisons of each of the methods were 0.85 ± 0.14 and 0.83 ± 0.10 for the July 2000 and July 2001 experiments, respectively.

Graphs of cell concentrations versus time at port 3 of MLS S10 for the July 2000 field experiment are presented in Fig. 3. Bromide concentrations are plotted as normalized values, as C/C_0 (where C is the concentration of Br in the sample and C_0 is the concentration of Br in the injection solution). Results obtained by the different enumeration methods were analyzed by Student's t test for two samples, assuming unequal variances. There were no statistically significant differences in the enumeration of TAMRA/SE-stained DA001 cells obtained by direct counts, microplate spectrofluorometry, and flow cytometry, whereas the ferrographic capture results were statistically

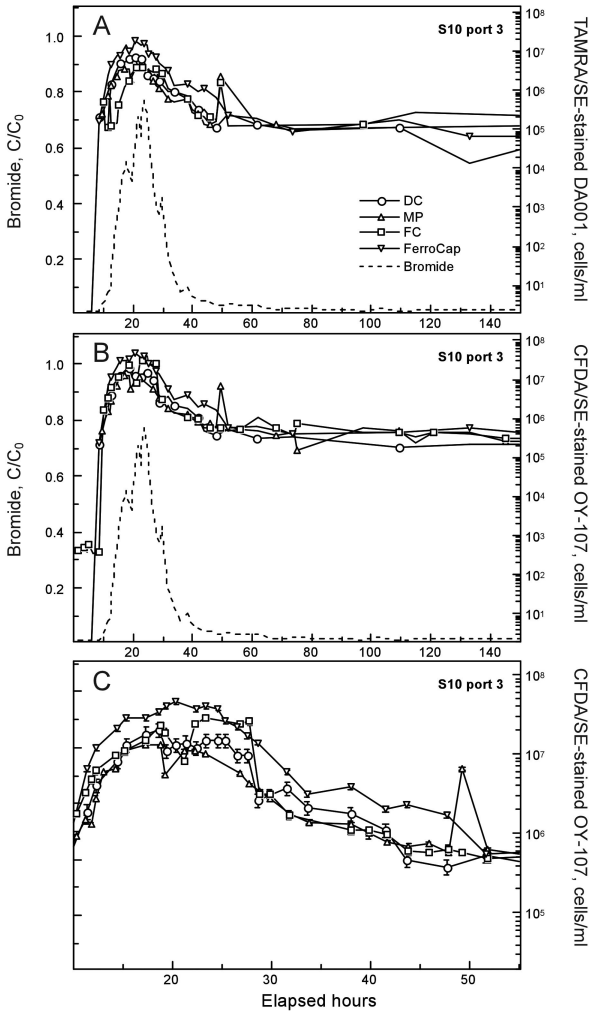

FIG. 3. Comparison of direct counts (DC), microplate spectrofluorometry (MP), flow cytometry (FC), and ferrographic capture (FerroCap) for enumeration of injected cells during the July 2000 field experiment. Samples were collected from port 3 of MLS S10. (A) TAMRA/SE-stained DA001 cells. (B) CFDA/SE-stained OY-107 cells. (C) Blowup of main peak area of the breakthrough curve, with error bars representing 1 standard deviation for the microplate spectrofluorometry data points and the previously determined error for direct count, flow cytometry, and ferrographic capture data points. Bromide data (dashed lines) are presented as normalized concentration profiles (C/C_0). Markers are shown for only 50% of the data points in panels A and B to make the plot more readable.

different (higher) from direct counts, microplate spectrofluorometry, and flow cytometry ($P = 0.0282$, 0.0008, and 0.0003, respectively). There was agreement between direct counts, microplate spectrofluorometry, and flow cytometry for the CFDA/SE-stained OY-107 cells (i.e., no statistically significant differences). Ferrographic capture results were marginally statistically higher than direct counts and microplate spectrofluorometry ($P = 0.0987$ and 0.0529, respectively) but were not significantly different from the flow cytometry results. The discrepancies between ferrographic capture and the other methods were possibly due to the ability of ferrographic capture to enumerate weakly stained cells (unpublished data). However,

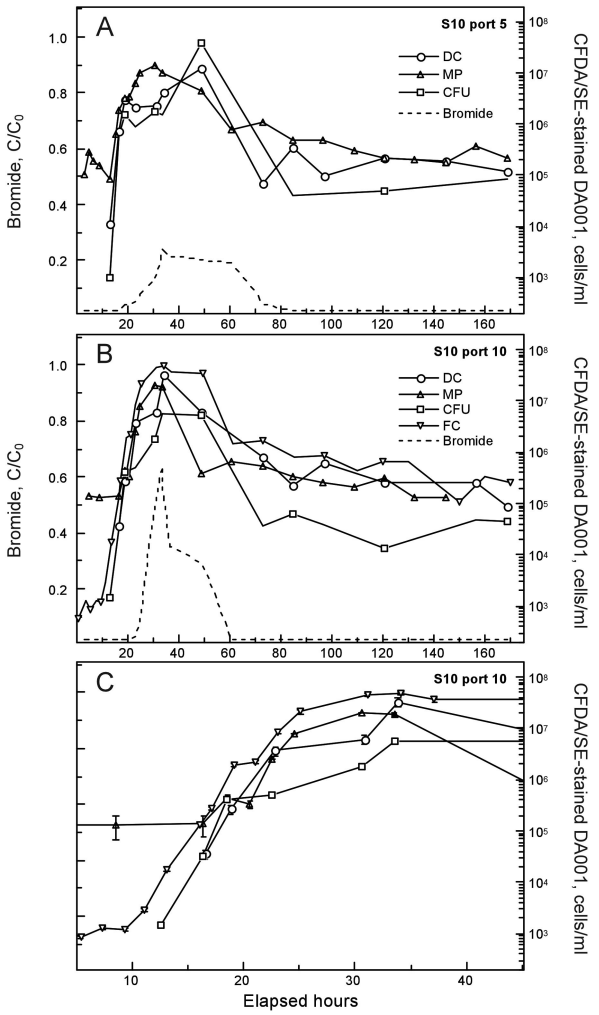

FIG. 4. (A) Comparison of direct counts (DC), microplate spectrofluorometry (MP), and spread plating (CFU) for enumeration of injected CFDA/SE-stained DA001 cells in samples collected from port 5 of MLS S10 during the July 2001 field experiment. (B) Comparison of direct counts, microplate spectrofluorometry, spread plating, and flow cytometry (FC) for enumeration of injected CFDA/SE-stained DA001 cells in samples collected from port 10 of MLS S10 during the July 2001 field experiment. (C) Blowup of main peak area of the breakthrough curve for port 10 of MLS S10, with error bars representing 1 standard deviation for the microplate spectrofluorometry data points and the previously determined error for direct count, CFU, and flow cytometry data points. Markers are shown for only 50% of the data points in panels A and B to make the plot more readable. Bromide data (dashed lines) are presented as normalized concentration profiles (C/C_0).

no further effort was made to determine the exact reason for these differences given the general agreement in the trends of bacterial breakthrough.

For the July 2001 field experiment, representative breakthrough curves were plotted as cell concentrations versus time for CFDA/SE-stained DA001 by direct counts, microplate spectrofluorometry, and CFU at MLS S10 port 10 (Fig. 4A), with flow cytometry results from port 5 of MLS S10 also included (Fig. 4B). There were no statistically significant differences between the results obtained with the three methods of enumerating stained cells or between the plate count method for culturable cells and the stained cell enumeration methods. At both depths in MLS S10, the apparent peak of viable cells, as determined by CFU, occurred slightly later than the peak of stained cells, as determined by microplate spectrofluorometry.

The results indicate that the overall assessment of bacterial transport was comparable by the different enumeration methods. Any of the methods would have yielded similar information about the vertical and horizontal movements of the injected bacterial plume. The calculation of the center of mass of the plume and relative breakthrough of the bacterial cells versus those of the conservative bromide tracer also resulted in comparable results (data not shown).

Near-real-time monitoring of *Comamonas* sp. strain DA001 and *Acidovorax* sp. strain OY-107 transport. The staining methodology combined with microplate spectrofluorometry performed in the field proved to be a robust and effective means to track the movement of bacterial plumes in near real time. The average delay between sample collection and sample analysis was approximately 1 h, although somewhat longer lags occurred during the initial 48 h due to the large number of samples collected. For both CFDA/SE- and TAMRA/SE-stained cells, the relative standard deviation for microplate spectrofluorometry analysis of four replicates of a single sample was 15% ± 6%, and only 1.5% of the analyses had relative standard deviation values of >50%.

Figure 5 presents the data from the July 2000 experiment from all of the MLSs along the centerline of the flow cell after krigging (or interpolation) and contouring at discrete times. It is apparent that the Br tracer and TAMRA/SE-stained DA001 cells exhibited a more conservative transport behavior than did CFDA/SE-stained OY-107 cells. It was also apparent from this time series of aqueous concentrations that most of the injected Br and TAMRA/SE-stained DA001 cells passed through the flow cell, whereas CFDA/SE-stained OY-107 cells were retained within the flow cell to a higher degree.

Vertical profiles of Br and both bacterial strains along the centerline of the flow cell during the July 2001 field experiment are shown in Fig. 6. Neither organism was detected by microplate spectrofluorometry analysis in MLS S24 by the end of the experiment, so contours were truncated at the position of MLS T2 (4.5 m down-gradient from the injection well). Cells likely passed entirely through the flow cell, as they did during the July 2000 experiment, but they were at concentrations below the microplate spectrofluorometry analysis detection limit during the July 2001 experiment. The abrupt truncation of the plumes in Fig. 6 is an artifact of the interpolation (krigging) method used to generate the plots. Given that TAMRA/SE-stained OY-107 cells were injected into the lower permeability depth interval of the aquifer during the July 2001 experiment, microplate spectrofluorometry analysis only sporadically detected this organism farther than 1 m down-gradient from well B2. This is reflected in the vertical profiles of OY-107 by the ovoid plumes of cells generated by krigging. In contrast, despite DA001 cells having been injected into a narrow depth interval in well B2 (spanning 3.5 to 4.5 m below msl), DA001 cells were detected in all ports (3.0 to 6.0 m below msl) in the down-gradient MLSs. The down-gradient dip of the DA001

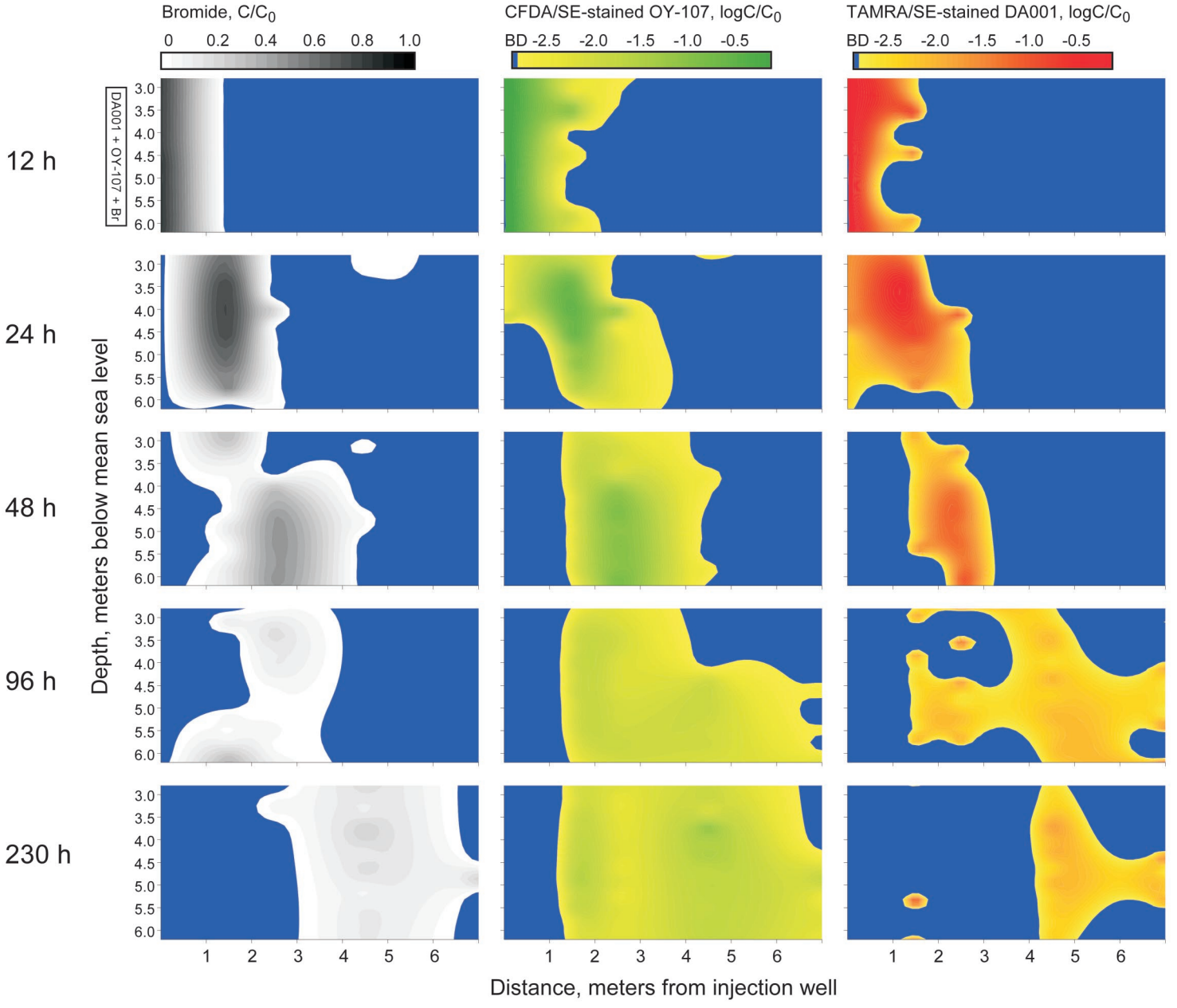

FIG. 5. Vertical profiles of TAMRA/SE-stained DA001 and CFDA/SE-stained OY-107 cells along the centerline of the flow cell during the July 2000 experiment, as determined by near-real-time microplate spectrofluorometry analysis. The injection interval is indicated by the vertical rectangle along the upper left plot. GW, groundwater.

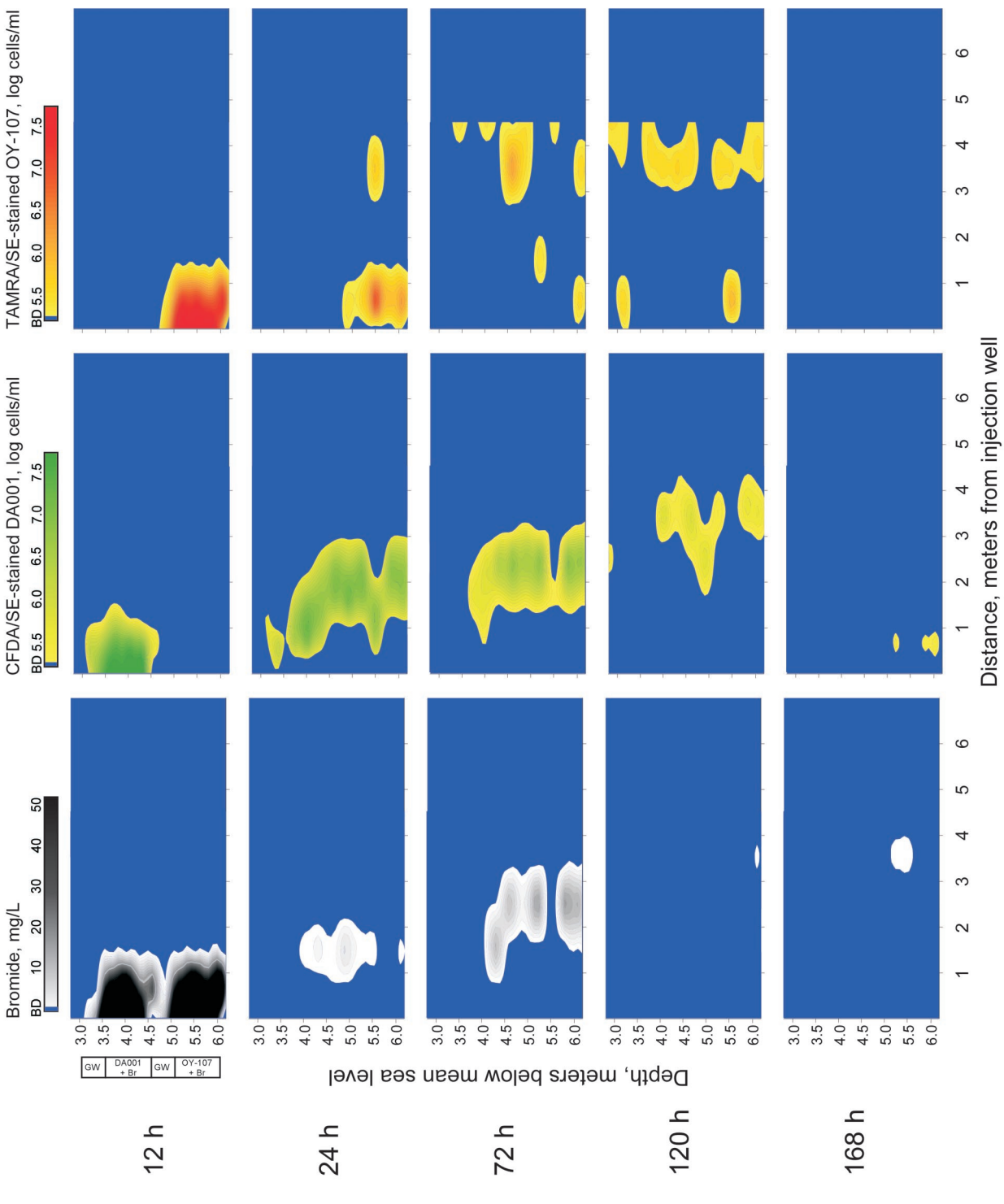

FIG. 6. Vertical profiles of CFDA/SE-stained DA001 and TAMRA/SE-stained OY-107 cells along the centerline of the flow cell during the July 2001 experiment, as determined by near-real-time microplate spectrofluorometry analysis. The injection interval is indicated by the vertical rectangle along the upper left plot. GW, groundwater.

plume followed the dip of the high-permeability zone present in the SOFA flow cell (compare Fig. 2 and 6).

The enumeration of stained cells was also performed in near-real time for the MLSs that were not on the centerline during both the July 2000 (S8 and S12) and July 2001 (S1, S3, S5, S6, S7, and S12) experiments. One common observation during both experiments was that higher cell densities and a somewhat earlier arrival of cells were detected in the wells south of the centerline wells (data not shown). Because this observation was made in the field in near-real time, it was possible to actually check the operational parameters (pumping flow rates, water elevations, etc.) as well as the sampling equipment to ensure that the plume of injected cells did not drift off-center and out of the flow cell due to some experimental error. All operational parameters and sampling hardware behaved as designed, and cells were eventually detected in the centerline MLSs at locations further down-gradient. Therefore, the cause of the southward plume drift at intermediate distances in the flow cell can be attributed to the hydrostratigraphy of the site. This highlights key advantages of the vital staining methods described here, namely the abilities to monitor in near-real time the movement of bacterial cells in the subsurface and to check during the experiment for artifacts that could potentially contribute to an unexpected transport behavior of injected microbes.

Conclusions. The research described here has demonstrated the feasibility of using the vital fluorescent stains CFDA/SE and TAMRA/SE to monitor coinjections of two different bacterial strains in the subsurface in near-real time. While this method is only explicitly recommended for the monitoring of cell concentrations under no-growth conditions, additional experiments published elsewhere indicated that CFDA/SE-stained cells can be detected and enumerated even after they have divided up to four times (13). As flow cytometers become less expensive and more portable, the resolution of monitoring concentrations of CFDA/SE-stained cells could be enhanced by taking one of these instruments to the field to perform the analyses. Additionally, the use of down-hole fluorometers with data-logging capabilities could further reduce sample processing and would be ideal for studies requiring an increased temporal data density. This vital staining method should therefore be applicable not only for answering questions in the area of subsurface bacterial transport, but also for examining other microbial ecology questions in both the laboratory and the field.

These results also highlight the utility of having multiple effective detection methods for enumerating cells released into the environment. The ferrographic capture and flow cytometry (and in some cases, plate counting) methods possess much lower detection rates than does microplate spectrofluorometry. This may be important for studies in which the detection of very low concentrations of cells is expected in the collected samples or the detection of even a few cells would be deemed significant (i.e., transport of pathogens). However, while microplate spectrofluorometry has a higher detection limit, the high sample throughput rate and the ability to perform analysis in near-real time in the field makes this enumeration method ideal for large-scale field experiments for which a good assessment of transport of the majority of the introduced cells is the final goal (as was the case with the field experiments described herein).

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