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Comparison of methods for monitoring bacterial transport in the subsurface

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Abstract

The purpose of this study was to compare in a laboratory experiment, a suite of methods developed to track viable bacteria during field transport experiments. The criteria for development and selection of these methods included: (1) the ability to track bacteria within the environment from which they were isolated; (2) the lack of any effect upon the viability or the transport characteristics of the strain; (3) low detection limits; (4) a quantification range that covered several orders of magnitude; and (5) an analytical cost and turnover time commensurate with the analysis of several thousands of samples in a few months. The approaches developed included: enumeration of bacteria labeled with a vital fluorescent stain (CFDA/SE) using microplate spectrofluorometry, flow cytometry, and ferrographic (immunomagnetic) capture; enumeration of highly ^{13}C-enriched bacteria using combustion-IRMS; and quantitative PCR. These methods were compared to direct microscopic enumeration and plate counts during a bacterial transport experiment performed in an intact sediment core and designed to simulate the field experiment. Four of the seven methods had equivalent recoveries for the breakthrough of a pulse of bacteria eluting from a 50-cm long sediment core, and all of the methods detected the arrival of cells in the effluent prior to the conservative tracer. Combustion IRMS and ferrographic enumeration had the lowest quantification limits ($\sim$2 to 20 cells/ml), whereas microplate spectrofluorometry had the highest quantification limit ($\sim 10^5$ cells/ml). These methods have the potential for numerous applications beyond tracking bacteria injected into the subsurface.

Keywords: Bacterial detection and quantification; In situ bacterial tracking; Viable stains

1. Introduction

Bacterial transport is of increasing interest to those involved in the remediation of contaminated subsurface environments because adding and dispers-

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ing bacteria that can degrade or transform contaminants (bioaugmentation) is an attractive and viable remedial option (Hinchee, 1995; Steffan et al., 1999; Caccavo et al., 1996; Gorby et al., 1994). However, the practice of bioaugmentation is limited by our knowledge of the factors that influence bacterial transport at the field scale.

Previous bacterial transport experiments have utilized various methods for tracking the introduced strain. In several transport experiments conducted in an aquifer on Cape Cod (Harvey et al., 1989, 1993, 1997; Scholl and Harvey, 1992; Bales et al., 1995; Harvey and Garabedian, 1991), indigenous groundwater bacteria were labeled with 4,6-diamidino-2-phenylindole (DAPI), a DNA-specific stain. This stain, which intercalates with the DNA of the cell, is known to affect the physiology of at least some bacterial strains (Parolin et al., 1990), and its effect on transport is also likely to be strain specific. Although DAPI did not appear to change the transport characteristics of one bacterial strain during a short-term laboratory experiment (Kucukcolak et al., 1998), its effect upon transport in a long-term experiment has yet to be assessed. The results of these experiments, therefore, may not be applicable to bioaugmentation strategies where metabolically active bacteria are injected into a contaminated aquifer.

In a number of cases, non-indigenous strains injected for bacterial transport or bioaugmentation studies have been readily enumerated by plate counts, either because the colonies could be readily distinguished visually from the background community (Champ and Schroeter, 1988; Dybas et al., 1998), or because selective agents (e.g., antibiotics) could be used to inhibit the background community from growing on the plates (Nelson et al., 1990; Steffan et al., 1999; Pang et al., 1997). During a previous bacterial transport study in a shallow, sandy aquifer near Oyster VA, selective plate counts were not sufficiently sensitive to detect the low numbers of cells that were transported (DeFlaun et al., 1997). However, combustion ^{13}C-IRMS analysis was successful in detecting the breakthrough of small numbers of ^{13}C-labeled bacteria during the same experiment (Holben and Ostrom, 2000).

Molecular methods have also been used for tracking bacteria during field experiments, including quantitative polymerase chain reaction (qPCR) methodology (Burlage et al., 1995; Thiem et al., 1994). Detection of the injected strains in these studies, however, was more qualitative than quantitative.

Tracking methods being developed for bacterial transport experiments planned for a field research site near Oyster, VA, were constrained by several requirements. Some of these requirements were mandated by the owners of the site, whereas others were necessitated by the potential future use of the methods for tracking cells during actual bioaugmentation activities. Constraints imposed on bacterial injection experiments at the South Oyster site required that the tracking methods used during those experiments: (1) be compatible with the use of a bacterial strain indigenous to the site; (2) cause no adverse effects on the physiology of the injected cells; (3) not involve the use of radioactive or toxic compounds; and, (4) cause no adverse affects on the transport characteristics of the cells.

Due to these constraints, several different tracking strategies were pursued. One tracking strategy employs vital fluorescent stains that exhibit no deleterious effects on the "fitness" or transport characteristics of the bacteria (Fuller et al., 2000a), as opposed to the DNA intercalating stains like DAPI. Quantification of the stained cells can be performed by either direct microscopic enumeration, microplate spectrofluorometry, flow cytometry, or ferrographic (immunomagnetic) capture (Zhang and Johnson, 1999). A second tracking strategy employs quantification of ^{13}C-enriched cells. Quantification of the cells can be performed by using whole cell combustion-IRMS (Holben and Ostrom, 2000) or HPLC-electrospray ionization-MS (Lytle et al., 2000). A final approach being explored is the further development of quantitative PCR. Although this method does not distinguish the injected cells from indigenous cells of the same species, it can be used effectively where background numbers of the bacterium of interest are low.

In this paper, we present a laboratory-scale bacterial transport experiment that was performed to directly compare the various tracking methods under conditions that were as close as possible to those expected during the planned field-scale bacterial transport experiments. The experimental design enabled the effects of sample transportation and holding time prior to analysis to be determined, as well

as a comparison of the results obtained by each method for identical samples.

2. Experimental

2.1. Chemicals and media

CFDA/SE (5-(and-6-)-carboxyfluorescein diacetate, succinimidyl ester), a vital fluorescent stain, was purchased from Molecular Probes (Eugene, OR, USA), and dual-labeled sodium acetate ($^{13}CH_3$ $^{13}COONa$, 99 atm%) was purchased from Isotec (Miamisburg, OH, USA).

Artificial groundwater (NCAG), based on groundwater chemistry of the Narrow Channel Focus Area at the South Oyster site contained (mg/l): $Ca(NO_3)_2 \cdot 4H_2O$, 70; $MgSO_4 \cdot 7H_2O$, 60; $NaHCO_3$, 60; $CaCl_2 \cdot 2H_2O$, 29; $CaSO_4 \cdot 2H_2O$, 25; KNO_3, 10; NaH_2PO_4, 0.4. NCAG was prepared by adding these salts to autoclaved distilled/deionized water and adjusting the pH to 6.0 with 2 N HCl. NCAG was sterilized by filtration through 0.2-μm cellulose acetate filter units.

2.2. Bacterial strain

An indigenous aquifer bacterium was isolated from the South Oyster field site and designated DA001. DA001 was identified as belonging to the genus *Comamonas* sp. based on phylogenetic analysis of its 16S rRNA gene sequence. It was most closely related to *Comamonas testosteroni*, with a sequence similarity of 96.9% over 1369 bases compared. An adhesion-deficient variant of DA001 was obtained by a simple column assay (DeFlaun et al., 1999), using Oyster site sediment. This variant adhered to site sediment in the column assay at $\sim 30\%$, while the parental strain adhered at $> 90\%$.

2.3. Preparation of bacteria

The bacteria injected into the core needed to be labeled in a way that facilitated enumeration by all the methods being tested. Therefore, 90% of the injected cells were stained with CFDA/SE and 10% of the cells were isotopically enriched with ^{13}C.

To maximize the degree of ^{13}C enrichment, DA001 cells were subjected to multiple rounds of culturing in which ^{13}C labeled sodium acetate served as the sole source of carbon and energy. A single colony of DA001 was inoculated into 1 ml of basal salts medium (BSM; DeFlaun et al., 1999) containing 0.2% w/v of uniformly ^{13}C-labeled ($> 99\%$) sodium acetate. After 1 week of incubation at room temperature (RT) with shaking at 600 rpm, the culture was serially diluted. One hundred microliters of the dilution (containing approximately 100 to 1000 cells/ml) was used to inoculate 1 ml BSM with 0.2% sodium acetate. A subsample of the culture was also plated onto R2A agar to determine the number of cells added to the fresh medium. The above procedure was repeated eight times prior to the intact core experiment to produce the starter culture for the experiment. Immediately preceding the experiment, the starter culture was grown with 0.2% sodium acetate in BSM without nitroloacetic acid (NTA-free BSM) to preclude a contribution of ^{12}C from the NTA. Following growth overnight at RT with shaking (150 rpm), 100 μl of this culture was used to inoculate two 10 ml volumes of 0.2% uniformly ^{13}C-labeled sodium acetate in NTA-free BSM. These cultures were grown at RT overnight with shaking at 150 rpm, pelleted by centrifugation (3400 rpm, 30 min, 4 °C), washed with NCAG, pelleted again and resuspended in 20 ml NCAG. This culture was held at 15 °C for 72 h with shaking (150 rpm). The cells were again pelleted and washed in NCAG, and finally suspended in 20 ml of NCAG prior to being mixed with CFDA/SE-stained cells.

A starter culture for the fluorescent staining procedure was prepared by growing DA001 in NTA-free BSM containing 0.2% sodium acetate overnight at RT with shaking. Six hundred microliters of this culture was used to inoculate 120 ml of NTA-free BSM containing 0.2% sodium acetate which was then incubated overnight at RT with shaking. The cells were pelleted (as described above), washed in 120 ml phosphate buffered saline (PBS), and resuspended in a final volume of 12 ml PBS. Staining of DA001 cells with CFDA/SE was performed as described in Fuller et al. (2000b), except that staining was performed during incubation in a 39 to 41 °C

water bath with continuous stirring for 2.25 h. After staining, the cells were pelleted, resuspended in 12 ml PBS and returned to the water bath for an additional 30 min. Cells were again pelleted, resuspended in 40 ml NCAG, and held at 15 °C for 72 h with shaking. The cells were then washed in NCAG and resuspended in a final volume of 20 ml of NCAG.

The stained culture (14 ml) was mixed with an aliquot of the ^{13}C-labeled cells (6.4 ml), 1.2 ml of 50 mg/ml bromide (as NaBr) and Oyster site groundwater to a final volume of 1200 ml (final Br concentration 50 mg/l). Of this volume, 750 ml was used as the core inoculum, with 705 ml being injected into the core and the rest used for plate count enumeration, direct microscopic enumeration and an assay to determine percent adhesion of the injected cells to site sediment as described previously (DeFlaun et al., 1999). The remaining volume of cells was used to prepare a set of serial dilutions in triplicate in NC site groundwater, yielding nominal cell densities of 10^0 to 10^8 cells/ml. These dilutions were preserved and stored in the same manner as the core effluent samples described below and used to generate standard curves for three of the tracking methods (microplate spectrofluorometry, qPCR, and ^{13}C-IRMS).

2.4. Intact core experiment

The intact sediment core (70 cm long × 7.2 cm diameter) used in this study was collected from the saturated zone during an excavation at the Narrow Channel Focus Area of the South Oyster site. Core orientation was parallel to the in situ groundwater flow. Core set-up (Fig. 1) and use were as described in DeFlaun et al. (1999), as modified in Fuller et al. (2000a), with the following further modifications. The plenum at the bottom of the core was eliminated and replaced with four inlet ports to insure that cells were introduced uniformly across the entire cross-section of the core. Core construction was simplified by sealing the top and bottom to the core with o-rings and threaded steel rod rather than silicon sealant. The bacterial transport experiment was conducted in a 15 °C controlled-environment room to closely approximate the in situ groundwater temperature.

The hydraulic conductivity in the core was calculated utilizing Darcy's law and constant head manometers (Wolf et al., 1991). Accuracy of the manometers was ± 0.5 mm and the difference between two consecutive calculations was less than 1%. Hydraulic conductivity was 1.47×10^{-4} m/s and the permeability was 3.21×10^{-1} Darcy.

An injection of ^3H_2O (30,000 dpm/ml) was performed before the cell injection at the same flow rate. The tritium tracer concentration in each fraction of the core effluent was determined to generate a tracer breakthrough curve. Briefly, 0.5 ml of each effluent fraction was added to 4.5 ml of Liquiscint scintillation cocktail (National Diagnostics, Atlanta, GA), vortexed and analyzed using a Model 1209 Rackbeta liquid scintillation counter (Pharmacia LKB Nuclear, Gaithersburg, MD). This resulted in a time-history of tracer elution from the core in terms of dpm/ml, which was converted to C/C_0 ((dpm/ml of eluted fraction)/(dpm/ml of injected solution)). The data from this injection was used to determine the porosity and dispersivity of the core by visual best fit of the non-retarded form of the advection–dispersion equation to the ^{3}H breakthrough curve. The best fit porosity and dispersivity were 0.38 and 0.85 cm, respectively. These values for porosity and dispersivity were validated by a high fit coefficient (0.99), calculated using the method of Hornberger et al. (1992).

The one pore volume (PV; 705 ml) inoculum was pumped into the core with an average volumetric flow rate of 1.05 ml/min resulting in a flow velocity of 0.99 m/day. After the cell injection was complete, the core influent tubing was switched to NC groundwater and an additional 6 PV was pumped through the core. In order to minimize pH changes that occur as the site groundwater equilibrates with the atmosphere, the site groundwater reservoir was continuously stirred and the pH was adjusted to 6.0 ± 0.2 S.U. with the addition of small amounts of 2 N HCl as needed. The duration of the experiment was 79 h, which equated to seven pore volumes through the core. Core effluent was collected at 20-min intervals using a fraction collector. The fractions were processed on a daily basis, and the pH of every fifth fraction was measured at the time of collection. The effluent was then processed immediately as described below.

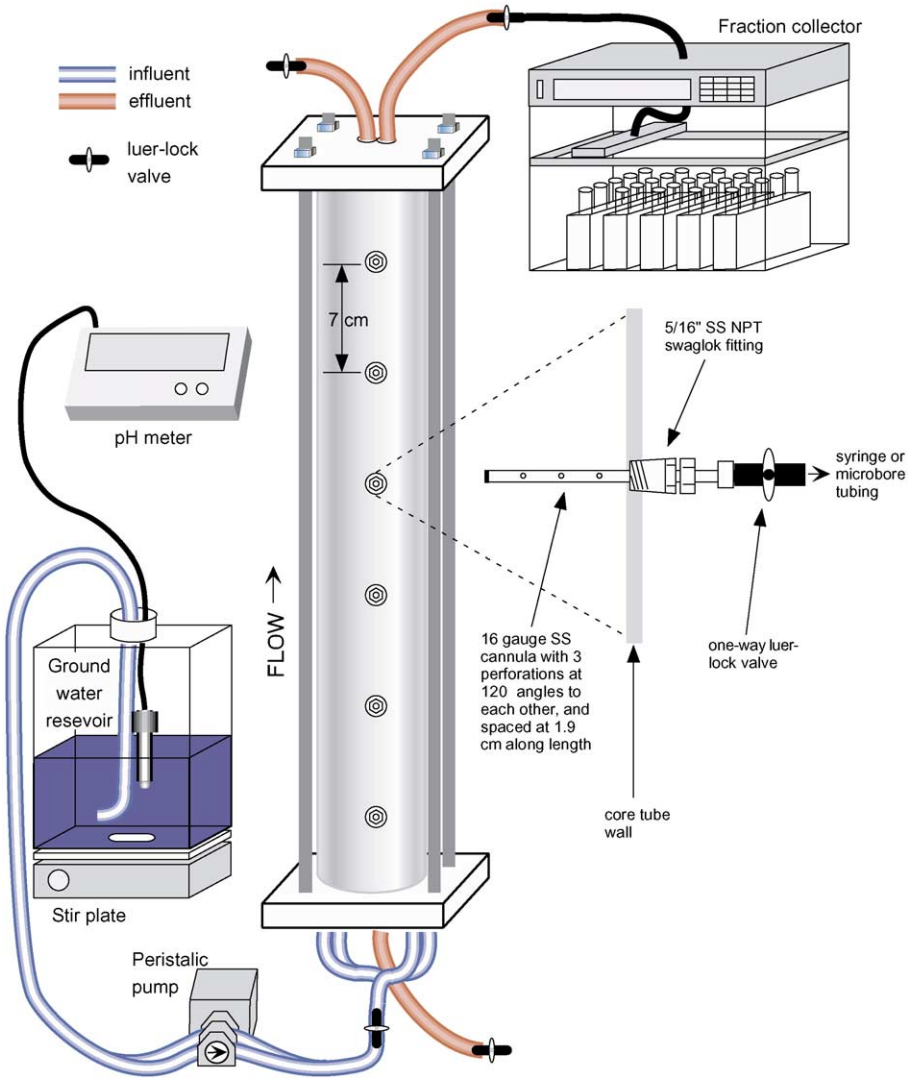

Fig. 1. Schematic diagram of the intact core experimental set up.

2.5. Sample processing

Core effluent fractions were pooled to achieve sample volumes of approximately 150 ml. Aliquots of effluent (150 ml) were split for analyses by the various tracking methods that were compared in this study. One aliquot (50 ml) was stored at 4 °C until the conclusion of the experiment and shipped on ice for qPCR and ^{13}C/IRMS analyses. Another aliquot (10 ml) was preserved with formaldehyde (1% final concentration; v:v), stored at 4 °C, and shipped on ice for ferrographic capture. A third aliquot (35 ml) was preserved with formaldehyde and stored at 4 °C for the following analyses: direct microscopic enumeration of CFDA/SE-stained cells (DC); fluorescence measurement using microplate spectrofluorometry (MP); flow cytometry (FC); and bromide measurement with an ion specific probe (Cole-Palmer 27502-05, Vernon Hills, IL, USA). A 1 ml unpreserved aliquot was analyzed using spread plating to determine the number of colony forming units (CFU) in each sample. Each method reported results in

cells/ml (or CFU/ml for spread plating), expressed as the average ± standard deviation when sufficient sample was available for multiple analyses.

2.6. Bacterial enumeration

2.6.1. Plate counts (CFU)

Samples were mixed well by vortex, then immediately serially diluted in PBS and plated in triplicate on R2A medium (Becton Dickinson, Cockeysville, MD). Plates were then incubated at 30 °C for 2 to 3 days, at which time colonies of DA001 were counted.

2.6.2. CFDA/SE direct microscopic enumeration (DC)

Direct microscopic enumeration was performed by filtering up to 10 ml of the formaldehyde-preserved samples onto a 0.2-μm polycarbonate black filter (Millipore, Bedford, MA). The amount filtered was calculated to deposit approximately 25 to 50 cells/field. Filters were mounted on slides with a drop of Slow-Fade Light antifade solution (Molecular Probes, Eugene, OR, USA). Cells were counted using a Nikon Labophot epifluorescence microscope equipped with a B-2H illumination block (Ex 470/20, Dichroic 510, Em 515) at 1250X. A total of 30 fields per slide were counted.

2.6.3. Microplate spectrofluorometry (MP)

Microplate enumeration of CFDA/SE-stained DA001 in core effluent samples was performed on formaldehyde preserved samples as described previously (Fuller et al., 2000b). The pH of the sample was raised to 8.0 using phosphate buffer (final concentration 0.01 M) to increase the fluorescence of the cells, thereby increasing the sensitivity of detection. Briefly, eight replicates (0.35 ml) of each well-mixed effluent sample were pipeted into a 96-well black microtitre plate (OptiPlate HTRF®-96, Packard Instrument, Meriden, CT, USA). The fluorescence of each well (Ex 495 nm, Em 538 nm, cutoff 530 nm) was determined using a Molecular Devices SPECTRAmax GEMINI dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA). This spectrofluorometer self-calibrates when started and before each microtiter plate is read. The average fluorescence signal of the eight replicates from each sample was converted to cells/ml using a standard curve generated by serially diluting the peak effluent sample, for which triplicate direct microscopic counts had been performed. The standard curve samples included a groundwater-only control which possessed some background fluorescence due to natural organic matter or other fluorescing agents in the groundwater. The fluorescence of this control was subtracted from the fluorescence of each of the standard curve samples before the best fit line equation was calculated. It was assumed that each core effluent sample contained the same amount of background fluorescence as the groundwater-only control. Therefore, the standard curve accounted for any background fluorescence in the effluent samples and no additional correction was applied.

2.6.4. Flow-cytometry (FC)

Enumeration of CFDA/SE-stained DA001 cells by flow cytometry was performed on formaldehyde fixed samples as described elsewhere (Fuller et al., 2000b), using a FACScan cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA), located at the Princeton University Flow Cytometry Core Facility. Prior to being introduced into the FACScan instrument, 1 ml of sample was mixed with 10 μl of 1.0 μm carboxylate-modified TransFluorSpheres (TFS, 1.8×10^4 microspheres/μl; Ex 488 nm/Em 645 nm, Molecular Probes, Eugene, OR, USA) to facilitate enumeration. Auto-calibration of the instrument occurs upon start-up. The detector settings were assessed at the start of each analysis run, and adjustments were made to assure that the measured fluorescent intensity of the TFS was the same for each run. Stained cells and TFS were excited with an argon laser tuned to 488 nm, with collection of emitted fluorescence from the cells and TFS at 515–545 nm (FL1) and 660–675 nm (FL3), respectively. All particles in a given sample were counted, with subsequent subtraction of the background particles that were too small and too faint to be bacterial cells or TFS. The same criteria for subtracting the background were used for both the core inoculum and all of the effluent samples. The number of TFS counted was used to calculate the volume of sample analyzed by the flow cytometer as described elsewhere (Blaß and Lennartz, 1991; Molema et al., 1998; Stewart and Steinkamp, 1982), since the FACScan instrument does not report this

value. Cell concentration in the sample was determined by dividing the number of stained cells counted by the sample volume analyzed.

2.6.5. Ferrographic capture (FerroTrack)

A polyclonal antibody was commercially developed (Rockland, Boyerstown, PA) to whole cells of *Comamonas* sp. strain DA001, and ferrographic capture was performed as described previously (Zhang and Johnson, 1999; Zhang et al., 1999). Ferrographic capture combines the selectivity of immunomagnetic tagging with the high resolution of ferrographic separation. During this analysis, antibodies conjugated to paramagnetic beads were allowed to interact with the DA001 cells in a given sample. The technique provides selectivity due to reliance on antibody–antigen recognition to magnetically tag the bacteria of interest. It also provides highly sensitive enumeration ($\sim$ 20 cells/ml for a 1-ml sample) due to deposition of the magnetically tagged bacteria or magnetic microspheres onto a small area (0.01 cm^2) of a glass microscope slide. The slide was then examined using an epifluorescence microscope to visually identify and enumerate the captured cells. Visual inspection also provided information on the relative shapes, sizes, and cell aggregation of the DA001 cells in the sample.

2.6.6. Stable carbon isotope ratio mass spectrometry (^{13}C-IRMS)

For stable isotope tracking by IRMS, the serial dilutions of the core inoculum were used to establish a relationship between δ^{13}C values and the number of ^{13}C-labeled bacterial cells in the presence of indigenous microorganisms and background ^{13}C in the particulate organic matter of the site groundwater. The dilution series and effluent samples were prepared and analyzed for stable isotope composition as described previously (Holben and Ostrom, 2000) except that a second-order polynomial regression analysis provided the best fit of the δ^{13}C values vs. total ^{13}C-labeled cell numbers. The regression equation was then used to convert the δ^{13}C obtained from each effluent sample to cell concentration.

2.6.7. Quantitative PCR (qPCR)

For enumeration of cells by qPCR, the relationship between a known number of cells (templates) and the amount of specific PCR product produced was determined. To accomplish this, a regression analysis was performed based on a serial dilution of known numbers of target cells and a fixed amount of competitor DNA. First, a dilution series of linearized competitor plasmid DNA was amplified alone to determine the linear range of detection and quantification of PCR product on ethidium bromide-stained agarose gels. The competitor DNA concentration corresponding to the midpoint of the linear range was then selected as the fixed amount of competitor for the regression analysis of known cell numbers, as well as analysis of samples with unknown numbers of cells. PCR reactions containing unknown numbers of cells were then performed and cells/ml values were calculated based on the regression formula. This approach is described in detail elsewhere (Kovacik and Holben, submitted for publication).

3. Results and discussion

Effluent concentrations as C/C_0 vs. time and pore volumes for the various tracking methods are compared to CFDA/SE direct counts (DC) and bromide tracer in Fig. 2. These comparisons are presented both on a log scale (Fig. 2A, C and E), as well as on a linear scale (Fig. 2B, D and F). The CFDA/SE direct count method only enumerated those cells added to the core which elute from the core as intact cells, and was therefore used for comparison to the other methods. Bromide (Br) recovery was 91% ($\pm$2), whereas the recovery of cells by the various tracking methods ranged from 93% ($\pm$12%) for plate count enumeration (CFU) to 23% ($\pm$1%) by ferrographic tracking. Percent recoveries for the CFDA/SE direct counts (DC, 45 ± 9%), flow cytometry (FC, 50 ± 10%), quantitative polymerase chain reaction (qPCR, 55 ± 11%), and ^{13}C-IRMS (55 ± 15%) were all within the range of 45% to 55%. These four methods yielded essentially equivalent recoveries of cells in the effluent given the associated errors and resulting standard deviations (DC, FC, qPCR and ^{13}C-IRMS). Microplate spectrofluorometry (MP, 28 ± 5%) and ferrographic capture (FerroTrack, 23 ± 1%) yielded lower recoveries, while CFU enumeration was much higher (93 $\pm$ 12%).

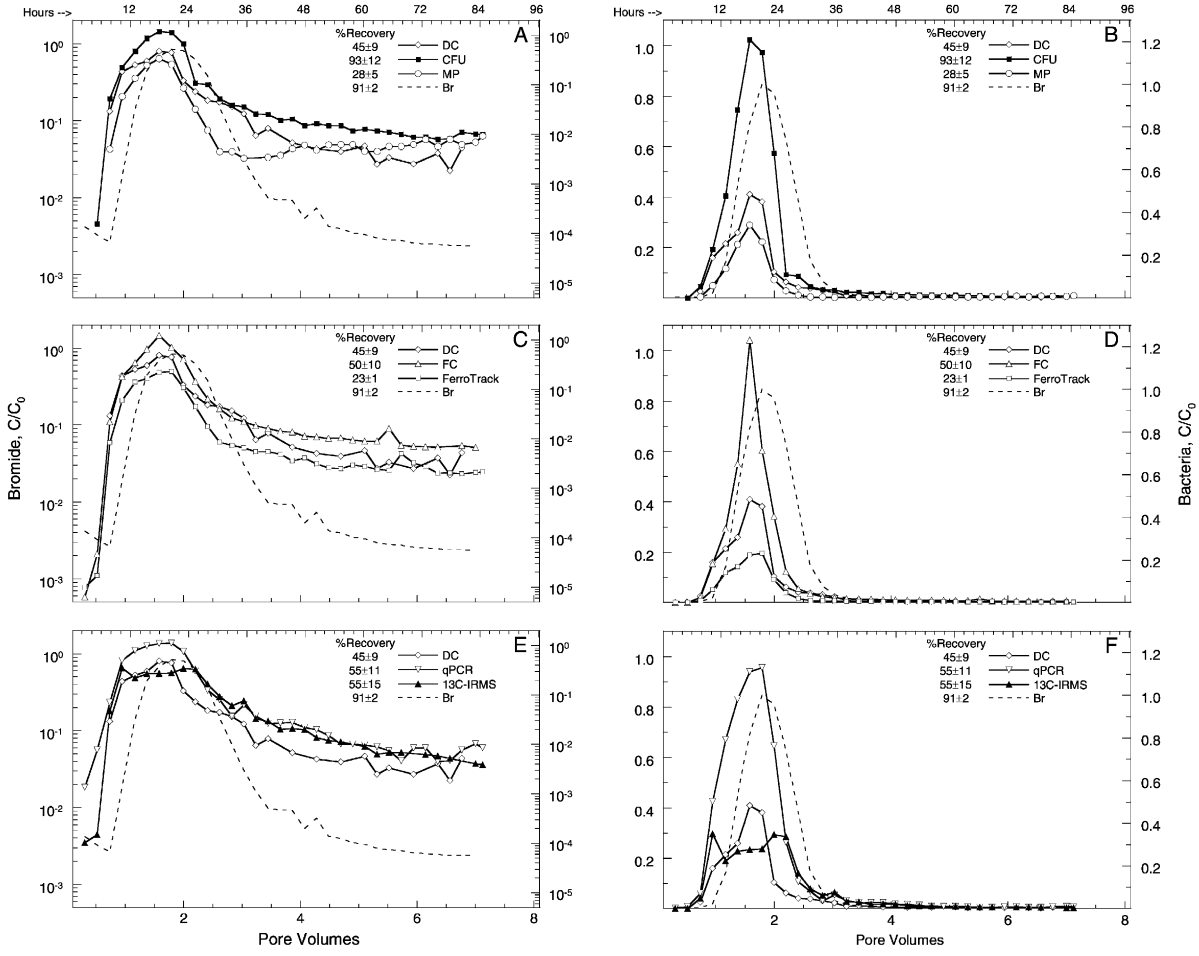

Fig. 2. Breakthough of conservative Br tracer (---) and DA001 cells, comparing results based on direct microscopic counts (DC, A) with: (A) plate counts (CFU, B) and microplate spectrofluorometry (MP, E); (B) same as (A) but linear scale; (C) flow cytometry (FC, C) and ferrographic capture (FerroTrack, G); (D) same as (C) but linear scale; (E) quantitative PCR (qPCR, S) and combustion–isotope ratio mass spectrometry (^{13}C-IRMS, H); (F) same as (E) but linear scale. Results are presented as concentration in a given sample/initial concentration injected into the intact core (C/C_0). Percents of the injected cells recovered in the effluent (avg ± error) based on each method are listed to the left of the legend.

Previous intact core transport experiments using *Comamonas* sp. strain DA001 labeled with radioactive tracers have shown 30% to 40% recovery of cells in the effluent. These results, as well as the potential for CFU counts to enumerate indigenous DA001-like organisms, indicate that the 93% recovery of DA001 in the effluent by plate counts in the present study is probably an overestimate of the actual recovery of the injected cells due to the presence of indigenous DA001 in the groundwater and sediment. Increases in indigenous cell numbers in the aqueous phase in response to a pulse injection of bacteria has also been observed during field injection experiments at the South Oyster site (Johnson et al., 2001b). The CFDA/SE direct count recovery (45%) is close to the range of effluent recovery previously seen for this organism in intact core experiments. All of the detection methods employed agreed fairly well as to the shape of the curve with the exception of ^{13}C-IRMS, which was flat in the point of the curve where the other methods showed a peak (Fig. 2E and F). This may indicate a high-end

limit of detection for this method under the conditions used in this study. Elution of DA001 cells before the bromide tracer was detected by all methods. Because of their lower limits of detection, the ferrographic capture and flow cytometry methods were able to detect DA001 earlier relative to the other methods (Fig. 2C). The ^{13}C-IRMS method should also have detected this early DA001 breakthrough due to its high sensitivity (as low as 2 cells/ml in a 50-ml sample). However, since only 10% of the injected cells were isotopically enriched with ^{13}C, the ^{13}C-IRMS method may have been compromised for low-end detection.

Correlation statistics for all of the methods comparing the C/C_0 vs. time plots (Fig. 2A, C and E) are presented in Table 1. Overall, qPCR had the highest correlations with all of the methods while ^{13}C-IRMS had the lowest correlations. Although FerroTrack had the lowest overall recovery, it had the highest correlation with direct counts ($r^2 = 0.96$). Conversely, ^{13}C-IRMS had the lowest correlation with direct counts ($r^2 = 0.50$), but its recovery was within the same range as DC. Plate counts (CFU), ^{13}C-IRMS, and qPCR generally overestimated cell numbers relative to direct counts, while ferrographic capture, microplate spectrofluorometry (MP), and flow cytometry (FC) generally underestimated cell numbers. The exception to this is at the lower end of the scale where both FC and MP tended to overestimate cell numbers with respect to direct counts.

The microplate spectrofluorometry method allows for large numbers of samples to be analyzed in a short period of time. The entire 96-well microplate, each well holding a separate sample or replicate, is read in approximately 30 s. Analyzing eight replicates of each sample results in a throughput of 60 samples per hour, including the time it takes to dispense the samples into the microplate. Also, the microplate spectrofluorometer is portable and can be used to measure the concentrations of bacteria in the field, an important consideration for field transport experiments. A potential disadvantage of this method is that microplate spectrofluorometry detects total fluorescence of the sample, which may or may not be cell-associated, this could result in overestimates of cell numbers. However, microplate spectrofluorometry results for this experiment appeared to underestimate the actual number of cells relative to direct counts (Fig. 2A and B).

The advantage of the flow cytometry method is that it analyzes each cell rather than just total fluorescence and therefore may be more specific than microplate spectrofluorometry. Flow cytometry data includes the concentration of stained cells, as well as information on fluorescence intensity per cell and the size and shape of the cells. The rate of analysis is slower than with microplate spectrofluorometry, but the limit of detection is also much lower (Table 2). Flow cytometry recovery results were similar to those from direct microscopic enumeration ($50 \pm 10\%$ vs. $45 \pm 9\%$; Fig. 2C and D). Analysis by flow cytometry can be subjective with respect to distinguishing between cells and other fluorescent particles based on the forward- and side-scatter, as well as the CFDA/SE fluorescence signal. With well-stained cells (as were used in this experiment), the selection of the cut-off is obvious. However, with cells not stained as well, distinguishing cells from other particles would require well-trained personnel.

Ferrographic capture has a very low limit of detection and does not require the use of a calibration curve. Unlike the microplate spectrofluorometry

Table 1
Correlation coefficients between the C/C_0 time profiles based on each method

	CFU	DC	MP	FC	FerroTrack	qPCR	^{13}C-IRMS
CFU	1.00						
DC	0.92 (26)	1.00					
MP	0.95 (31)	0.95 (25)	1.00				
FC	0.85 (32)	0.84 (26)	0.87 (30)	1.00			
FerroTrack	0.97 (33)	0.96 (26)	0.95 (31)	0.84 (33)	1.00		
qPCR	0.96 (33)	0.92 (26)	0.91 (31)	0.87 (33)	0.97 (34)	1.00	
^{13}C-IRMS	0.60 (29)	0.50 (24)	0.48 (27)	0.60 (29)	0.62 (30)	0.72 (30)	1.00

Values are presented as r^2 (n). Correlations were significant for all comparisons ($p < 0.0001$).

Table 2
Comparison of the methods with respect to several aspects of performance and cost

	CFU	DC[a]	MP[b]	FC[c]	FerroTrack	qPCR	^{13}C-IRMS
Cost of labeling ($)[d]	–[e]	2.48	2.48	2.48	2.48[f]	–	9.10
Cost per sample for ($)[g]							
Supplies	2.70	1.14	0.22	0.17	1.87	1.00	–
Analysis fees	–	–	–	1.14	–	–	35.00
Samples analyzed per hour[h]	3.48	1.45	60	35	5	30	2
Detection limit (cells/ml)	1×10^2	1×10^4	3×10^5	1×10^3	1×10^1	5×10^4	2×10^0
Sample volume required (ml)[i]	0.10	0.05–10	0.35	0.50–1.0	0.50–1.0	0.025	50
Reproducibility ($\pm$% of value)[j]	10	20	15	8	10	12	25

[a]DC, Direct microscopic count of CFDA/SE-stained cells.
[b]MP, Fluorescence microplate enumeration of CFDA/SE-stained cells.
[c]FC, Flow cytometric enumeration of CFDA/SE-stained cells.
[d]Labeling is the cost of chemicals for preparing the inoculum for one intact core experiment, approximately 1×10^8 cells/ml, 1 l total volume, excluding labor.
[e]–, Not applicable.
[f]The FerroTrack method can be used with unstained cells by employing a fluorescently labeled secondary antibody.
[g]Cost excluding labor and capital equipment.
[h]Time per sample includes pre-analysis preparation, analysis, and post-analysis calculations. Time for MP analysis assumes eight 0.35 ml replicates per sample were analyzed. Time for FC increases with very low concentration samples.
[i]Volume reported is for analysis of a single replicate of a sample. Time for FerroTrack assumes a single operator using epifluorescent microscopic enumeration of cells.
[j]Reproducibility expressed as the percent relative standard deviation of the mean of multiple measurements for a typical sample. Value decreases with increasing cell density above the detection limit.

method, it is based on direct quantification of captured cells. Optimal conditions for 100% recovery of low concentrations (100 cells/ml) of spiked bacteria (*Escherichia coli*) by ferrographic separation from a variety of synthetic and natural waters have been determined (Zhang and Johnson, 1999; Zhang et al., 1999). These studies have shown that detection of < 20 cells/ml is achieved by FerroTrack analysis of only 1 ml of sample. The most likely source of error in enumeration by ferrographic capture is incomplete immunomagnetic tagging of the microbes, which can potentially result from non-specific interaction of the primary antibody with colloidal materials (e.g. minerals) in the sample. The presence of suspended kaolinite colloids and dissolved cations in natural waters has been found to require the addition of skim milk (1%) or Bovine Serum Albumin (1 mg/ml) to block non-specific antibody interaction with colloids. Addition of ethylenediaminetetraacetic acid (EDTA, 2 mM) to some natural water samples prior to analysis was also required to mitigate cation-induced antibody coagulation during analysis (Zhang et al., 1999). Since this incomplete tagging is the most significant potential error, this method tends to underestimate rather than overestimate the actual bacterial concentration. This could explain the low recovery of this method compared to the others employed during this study.

The detection of high concentrations of cells ($>$ 5000 cells/ml) by ferrographic enumeration requires sample dilution prior to analysis, and cursory direct counts may be necessary to determine the appropriate dilution. Because this method relies on visual recognition of the cells, aggregation of the cells can be observed and accounted for. Once the cells are captured further analyses are possible, including rRNA probing and even cell culturing. While this method takes more time than flow cytometry (Table 2), the capital investment is much lower. Recovery by ferrographic capture was similar to that for the microplate spectrofluorometry method, and it also appeared to underestimate (by about a factor of two) the total number of cells in the core effluent compared to direct counts (Fig. 2D). However, ferrographic capture results from a subsequent field injection show excellent agreement with the other tracking methods (Johnson et al., 2001a). The use of fluorescent primary antibodies as linkages to the

magnetic beads provides an outline of the captured cells under epifluorescence, whereas fluorescent staining of the cell interior with a complementary fluorophore provides an alternative means of identification. The use of pre-stained cells in combination with the ferrographic capture allows selective enumeration of the added cells, which is important in an environment where the bacterium being added was originally isolated from the indigenous population. The use of fluorescent antibody in immunomagnetic tagging allows the indigenous and the added organism to be enumerated. This method also has the ability to simultaneously enumerate multiple strains, either by using different cell stains or by using different fluorophores on the primary antibodies.

The ^{13}C-IRMS method has a low limit of detection, and is specific for the injected organism. Holben and Ostrom (2000) have shown that the potential for label turnover and lysis is not significant in this system. The cost and time for each analysis, however, places limits on the number of samples that can be processed. Recoveries of DA001 during this experiment based on the ^{13}C-IRMS method were in the expected range (Fig. 2E and F). However, this method also had the greatest error associated with it (Table 2).

The qPCR method is rapid, inexpensive and amenable to automation. It involves a direct detection of chromosomal DNA of the organism of interest, so there are no concerns about the stability of the marker. The ability to employ multiple primer sets in a single reaction allows the simultaneous detection of multiple organisms. However, this approach does not discriminate between the injected cells and the indigenous background of the organism of interest. In other words, the qPCR method is specific for the organism of interest, but not for the injected population of that organism. Therefore, its application for tracking added organisms would be limited in settings where high indigenous numbers of the strain of interest are present. The values for cells/ml by qPCR were consistently higher than for direct microscopic enumeration, but the percent total recovery of cells in the effluent was similar for these two methods (Fig. 2E and F).

While all of the methods tested are potentially applicable to a wide variety of bacterial strains, qPCR and ferrographic enumeration require development of strain-specific reagents in preparation for their use. The other methods tested (i.e. vital staining with detection by microplate spectrofluorometry or flow cytometry, and ^{13}C-IRMS) require less a priori knowledge of the organism of interest to facilitate detection. Conversely, since no pre-injection "tagging" or labeling is required for detection by ferrographic capture and qPCR, these two approaches are the best suited of this suite of methods to detect and monitor the natural abundance of specific indigenous microbes (i.e. organisms of interest that have not been injected into the system).

All of these methods have applications beyond tracking cells during bacterial transport experiments or for monitoring bioaugmentation. For example, ferrographic capture combined with a vital stain such as CFDA/SE can be used to determine the response of an indigenous strain to the introduction of large numbers of cells of the same strain. Also, predation or turnover of introduced cells can be monitored by tracking the ^{13}C signature or CFDA/SE stain into higher organisms or by isotopic dilution.

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