Applied and Environmental Microbiology, July 2006, p. 4672–4687 Vol. 72, No. 7
0099-2240/06/$08.00+0 doi:10.1128/AEM.02858-05

Suspension Array Analysis of 16S rRNA from Fe- and SO_4^{2}-Reducing Bacteria in Uranium-Contaminated Sediments Undergoing Bioremediation

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Received 5 December 2005/Accepted 4 April 2006

A 16S rRNA-targeted tunable bead array was developed and used in a retrospective analysis of metal- and sulfate-reducing bacteria in contaminated subsurface sediments undergoing in situ U(VI) bioremediation. Total RNA was extracted from subsurface sediments and interrogated directly, without a PCR step. Bead array validation studies with total RNA derived from 24 isolates indicated that the behavior and response of the 16S rRNA-targeted oligonucleotide probes could not be predicted based on the primary nucleic acid sequence. Likewise, signal intensity (absolute or normalized) could not be used to assess the abundance of one organism (or rRNA) relative to the abundance of another organism (or rRNA). Nevertheless, the microbial community structure and dynamics through time and space and as measured by the rRNA-targeted bead array were consistent with previous data acquired at the site, where indigenous sulfate- and iron-reducing bacteria and near neighbors of *Desulfotomaculum* were the organisms that were most responsive to a change in injected acetate concentrations. Bead array data were best interpreted by analyzing the relative changes in the probe responses for spatially and temporally related samples and by considering only the response of one probe to itself in relation to a background (reference) environmental sample. By limiting the interpretation of the data in this manner and placing it in the context of supporting geochemical and microbiological analyses, we concluded that ecologically relevant and meaningful information can be derived from direct microarray analysis of rRNA in uncharacterized environmental samples, even with the current analytical uncertainty surrounding the behavior of individual probes on tunable bead arrays.

Nucleic acid technologies are now routinely used in environmental microbiology, and increasingly they contribute to our understanding of microbial diversity, abundance, or activity in the environment. In particular, microarray technology represents the next technological step beyond the PCR for highly multiplexed detection, and it is employed for pathogen detection (14, 35, 37), metagenomics (33), expression profiling of environmentally relevant bacteria (4, 25, 31), microbial taxonomy and species determinations (9, 27), understanding genome plasticity (10), microbial community profiling (11, 15, 23, 32), and identification of metabolically active microorganisms (1). Despite the rapid acceptance and adaptation of microarray methods in environmental microbiology, there are still a number of technology assumptions that are important when microarray data need to be converted into information so that a user can make a decision or take an action. In particular, how does one interpret microarray signals and make ecological conclusions when he or she is confronted with an uncharacterized environmental background and imperfect, variable, and noisy microarray data (6)?

The technological issues of microarray variability and cross-hybridization are not unique to environmental microbiology (19, 34, 41), but the technology-ecology intersection becomes acute, for example, when workers attempt to develop, apply, and regulate in situ bioremediation programs and make long-term stewardship decisions for contaminated subsurface environments. The U.S. Department of Energy Natural and Accelerated Bioremediation Program has established a number of field research centers in order to conduct fundamental environmental science and to understand how to translate knowledge into remedial action and stewardship decisions. The Uranium Mill Tailings Remedial Action (UMTRA) site in Rifle, CO, is one such field research center, and a number of geochemical, microbiological, and bioremediation studies have already been completed and published or are ongoing (3, 8, 12, 28). These studies have clearly shown that in situ bioremediation can be used to reduce and immobilize U(VI) in subsurface environments and that known genera of metal- and sulfate-reducing bacteria are present and active at the site. However, both near-term remediation and long-term stewardship decisions would benefit from a more accurate and timely assessment of microbial community composition and dynamics in response to accelerated or natural bioremediation.

To that end, we have been developing a suite of affinity purification techniques, renewable-surface fluidic systems, and bead array detection methods for automated isolation, purification, and direct detection of RNA from environmental samples (5). The Old Rifle site provides an excellent opportunity to integrate information on groundwater flow, sediment characteristics, geochemistry, and microbiology which has been described previously or will be described in the future. At the

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same time, the site data provide an excellent backdrop and context within which to ask some fundamental microarray technology questions. Namely, do the data make sense? And can we deconvolve microbial community profiles from potentially noisy microarray data and still provide meaningful information to bioremediation engineers? The objectives of this study were, therefore, to (i) develop and use a 16S rRNA-targeted bead array for analysis of metal- and sulfate-reducing bacteria in contaminated subsurface sediments; (ii) perform a retrospective analysis of UMTRA sediments before and after biostimulation; and (iii) determine if the resulting microbial community profiles "make sense" and convey meaningful information or insight regarding the underlying microbial community dynamics during in situ U(VI) bioremediation.

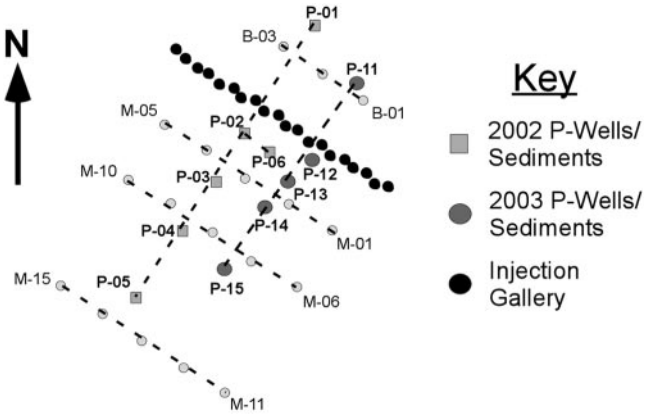

FIG. 1. Old Rifle site and sediment sample set. The background samples included B01 to B03, M03, M08, and M13. Samples P01 (2002) and P11 (2003) are the appropriate background and reference samples for comparing down-gradient 2002 and 2003 borehole samples P02 to P06 and P12 to P15; the 2002 and 2003 samples are connected by dashed lines. Monitoring wells are indicated by designations beginning with M and are connected by dashed lines. The acetate injection gallery was perpendicular to the borehole transects, as indicated.

MATERIALS AND METHODS

Old Rifle site description and sediments. The Old Rifle site layout and geochemistry have been described in detail elsewhere (3, 36), and the locations of sediment boreholes and monitoring wells used in 2002 and 2003 are shown in Fig. 1. Injection wells were installed to a depth of 6.1 m (20 ft) and screened from 1.5 to 6.1 m (~5 to 20 ft) to encompass the entire saturated interval of the aquifer. Acetate was injected during 2002 as described previously (3), which resulted in final acetate concentrations of 1 to 3 mM in the aquifer for each day of the treatment. Acetate was continuously injected into the Old Rifle aquifer for 80 days between June and October 2002. While acetate amendment was still under way, sediment samples were collected by rotary sonic drilling through the aquifer.

Based on microbiological and geochemical measurements obtained during 2002, the treatment regimen was modified for the 2003 field season by increasing the within-aquifer acetate concentration to 10 mM and increasing the duration of acetate injection to 100 days. Sediments for both 2002 and 2003 treatments were collected from depths of 13, 15, 17, and/or 19 ft (~4.0, 4.6, 5.2, and 5.8 m). Samples were processed aseptically in a nitrogen atmosphere inside a portable field glove bag, and individual aliquots were provided to several investigators. Large pebbles and cobbles were removed from the cores, and the remaining samples that were provided to investigators were dominated by particles that were sand to silt sized. While rotary sonic drilling disaggregated and mixed cores and the overall stratigraphic relationships were maintained from core to core, the desire to process samples as rapidly as possible limited the ability to deliberately homogenize sediment samples. Sample aliquots were immediately frozen and shipped on dry ice, and they were stored at −20°C or a lower temperature.

Oligonucleotide probes. The rationale, logic, and design strategy for oligonucleotide capture probes have been described in detail previously (7). Capture probe sequences were derived from alignments of dissimilatory metal and sulfate reducer 16S rRNA sequences deposited in the GenBank database, utilizing only full-length sequences for which an isolate is available in a public culture collection. All capture probes were designed by using the 420 region of the 16S rRNA (*Geobacter chapellei* numbering; accession number U41561) based on ClustalW alignments of more than 250 full-length 16S rRNA sequences for metal- and sulfate-reducing bacteria. The 420 region contains both variable and conserved sequences for the metal and sulfate reducers, which allowed us to design species-specific capture probes and genus (or higher taxonomic rank) chaperone detector probes using the chaperone hybridization and detection scheme described previously (7) and used here. Variable sequences in other regions of the 16S rRNA were not included in the bead array because of concerns about differential hybridization efficiency due to target accessibility (secondary, tertiary, and perhaps quaternary interactions) and because the standard Luminex bead set is restricted to only 100 colors (or probes). DNA capture probes contained a 5′ biotin and at least two mismatched nucleotides between all other capture probes on the array (Table 1), and we attempted to place the mismatches toward the central positions of the capture probes. All capture probes were synthesized in house with standard phosphoramidite chemistry using a MerMade IV DNA/RNA synthesizer (96-well format) and were purified by high-performance liquid chromatography. A series of chaperone (or helper) probes for disrupting the RNA secondary and tertiary structure in the target region were synthesized and purified by high-performance liquid chromatography at BioSource International (Camarillo, CA), and these probes targeted either the 420 region of the metal and sulfate reducer rRNAs (7) or higher-order tertiary (universal) rRNA structures (this study). Two nonsense probes (also containing a 5′ biotin) were synthesized (Sigma-Genosys, The Woodlands, TX) and used to correct for nonspecific background hybridization to the bead array for the sediment samples, as described below. All probe stock solutions were reconstituted in diethyl pyrocarbonate-treated water, the concentrations were adjusted to 200 μM, and the preparations were stored at −20°C until they were used.

Bead conjugation. Biotinylated DNA probes were coupled to Lumavidin-coated beads as specified by Luminex (Austin, TX). The efficiency of probe coupling to Lumavidin beads was determined by competitive binding experiments performed with biotin-phycoerythrin according to the Luminex instructions, and the values ranged from 69 to 95% of the available biotin sites for the validation array and from 61 to 99% for the Old Rifle sediment arrays. Conjugated beads were stored at 4°C, and all bead concentrations were verified by counting bead slurries in an improved Neubauer Brightline counting chamber (Hausser Scientific Co., Horsham, PA) before use.

Bead array analysis of bacterial strains. Twenty-four isolates of iron- and sulfate-reducing bacteria (Table 2) were used to prevalidate the bead array. *Geobacter* and *Desulfovibrio* isolates were cultivated as described previously (7); SMCC and OCM isolates were purchased as frozen cell pellets from Portland State University, and ATCC isolates were cultivated in the ATCC-specified media. Cells were harvested by centrifugation at 8,000 × *g*, and total RNA was extracted from cell pellets using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Extracted RNA was treated with DNase I according to the manufacturer's instructions (Ambion Inc., Austin, TX), purified by phenol-chloroform extraction, and ethanol precipitated overnight. Purified RNA was reconstituted in dimethyl pyrocarbonate (DMPC)-treated water and quantified by determining the UV absorbance, and the presence of 16S rRNA was confirmed by gel electrophoresis in 2% agarose and Tris-acetate-EDTA running buffer. Total RNA was then fragmented and labeled, and 100 ng was hybridized to the bead array for 2 h at 45C as described in detail elsewhere (5). At least three replicate hybridizations were performed for each isolate, and the data were used to calculate a median fluorescence intensity (MFI) (average ± standard deviation), which was adjusted for differential hybridization efficiency and nonspecific and cross-hybridization noise by subtracting the average bead array MFI from individual probe responses. The protocol used for extraction, fragmentation, labeling, hybridization, and analysis of sediment-derived RNA differed slightly from the previously described protocol (7), as described below.

Bead array analysis of Old Rifle sediments. Total RNA was extracted from 10 0.5-g aliquots of each sediment sample by bead beater extraction (BioSpec Products, Bartlesville, OK) in 2-ml cryo tubes containing 0.5 g of 0.1-mm glass beads and 800 μl of lysis buffer (4 M guanidine thiocyanate, 45 mM Tris-HCl [pH 6.4], 66 mM Na_4EDTA [pH 8.0], 1.1% Triton X-100, 65 mM $MgCl_2$) for 3 min at the maximum speed (~5,000 oscillations min^{-1}). The tubes were chilled briefly on ice and centrifuged at 12,000 × *g* at 4°C for 3 min to remove the beads and sediment. Supernatants from the 10 replicate aliquots were pooled, and the RNA was precipitated directly with ethanol by overnight incubation at room

TABLE 1. Microarray capture and chaperone/helper probes

Probe	Sequence (5′–3′)	Target(s)	GenBank accession no.
Capture probes[a]			
1	CCTATTCGAACGGTACTTGTT	*Bacillus subtilis*	AB018486
2	TACCCGCRACACCTAGT	*Geobacter* group[b]	
3	CATTATTTACATACTTACCGTT	*Desulfitobacterium frappieri*	DFU40078
4	CATTCTTTACATACTTACCGTT	*Desulfitobacterium hafniense* DCB-2T	X94975
5	ATCTGTTAAACAATTGTAACTT	*Desulfobacter postgatei*	AF418180
6	GATTATTCGTCCCTTGAGGGTT	*Desulfoarculus baarsii*	AF418174
7	TTCTATTAAAACATTGGAACTT	*Desulfobacter curvatus*	M34413
8	ATCTGTTAAACAATTGTAACTT	*Desulfobacter postgatei*	AF418180
9	TACTATTAATAGAAGCTAATTT	*Desulfobacter hydrogenophilus*	M34412
10	AGGTATTAACTACTATGCACTT	*Desulfobacter rhabdoformis*	U12253
11	GGGTATTAACCGCTATGCACTT	*Desulfobacter elongatus*	X95180
12	ATTCATAAGGTACCGTCATTAT	*Sulfurospirillum barnesii*	AF038843
13	CTTGCTGTTTACAAGAAATCTG	*Desulfotomaculum acetoxidans*	Y11566
14	TACTCTTCAGGTACCGTCATTT	*Desulfotomaculum reducens*	U95951
15	TCCTCGTTGGGTACCGTCACTT	*Desulfotomaculum thermobenzoicum*	Y11574
16	TACTCCTATGGTACCGTCACTT	*Desulfotomaculum halophilum*	U88891
17	TCTTAGTCAGGTACCGTCATTT	*Desulfotomaculum guttoideum*	Y11568
18	TCCTCCTAAAGTACCGTCATTT	*Desulfotomaculum nigrificans*	AB026550
19	TACTCCTATGGTACCGTCACTT	*Desulfotomaculum alkaliphilum*	AF097024
20	TCCTCTCCAAGTACCGTCATTA	*Desulfotomaculum putei* SMCC W464	AF053934
21	TCCTCCTGGAGTACCGTCACTT	*Desulfotomaculum luciae*	AF069293
22	ACTCAATATTTTCAAATCGACCG	*Desulfotomaculum orientis*	Y11571
23	TCTTGTTATTTACAAGAGAGCTG	*Desulfotomaculum* sp. strain ASRB-Zg	AJ276370
24	GCCTATTAGACCAATGCCCGTT	*Desulfomicrobium norvegicum*	AJ277897
25	GCTGATTAGCACAACGTAGTTT	*Desulfovibrio desulfuricans*	M34113
26	CTCTGTTCGAAACCTGGCGGTT	*Desulfovibrio sulfodismutans*	Y17764
27	GCTGATTAGCACCGTGGCGGTT	*Desulfovibrio vulgaris*	AF418179
28	CAAGCCTATTTGAAATGGCGCGTTT	*Desulfovibrio senezii*	AF050100
29	GAGTCGTATTAATACTCTACGGTTT	*Desulfovibrio dechloroacetivorans*	AF230530
30	CACTATTCGCATCCTCGGGGTT	*Desulfovibrio gabonensis*	U31080
31	ATTTATTAAATCTTATGTGGTT	*Desulfovibrio bastinii*	U53462
32	GGACCGTATTAATGTCCAACAGTTT	*Desulfovibrio caledoniensis*	U53465
33	GAGCCTATTCGACTCCAACAGTTTC	*Desulfovibrio profundus*	U90726
34	GCTGATTAGCACCATGGCGGTT	*Desulfovibrio longreachii*	Z24450
35	CGATATTAACACCCGGGAGTTT	*Desulfuromonas acetoxigens*	U23140
36	GGGTATTAGCCAACAAAGGTTT	*Desulfuromonas acetoxidans*	M26634
37	TGTTATTATCACCCGGGAGTTT	*Desulfuromonas chloroethenica*	U49748
38	GGATATTAACCTACAGGGGTTT	*Desulfuromonas palmitatis*	U28172
39	GTCAGCACTCTTGCGAGTATTT	*Desulfuromusa bakii*	X79412
40	AGGGTATTAACCCATGCGTTTT	*Ferribacterium limneticum*	Y17060
41	CGGGTATTAACCACTAACCTTT	*Ferrimonas balearica*	X93021
42	TGGATATTAGCCAGCCCCATTT	*Geobacter bremensis*	U96917
43	GGATATTAGCCACAATACATTT	*Geobacter* sp. strain CdA-2/3	Y19190
44	GGGTATTAACCAAGCTCATTT	*Geobacter* sp. strains Ala-5 and Ala-6	AF019928, AF019929
45	GGGTATTAACCAACAGAGGTTT	*Geobacter* sp. strain SBD-1	AF019933
46	GGATATTAGCCACAATACACTT	*Geobacter chapellei*	U41561
47	TGGTATTAGCACCCTTACACTT	*Geobacter humireducens*	AF019932
48	GGGTATTATCCCTCAATCATTT	*Geobacter hydrogenophilus*	U28173
49	GGGTATTAACCCTCAATCACTT	*Geobacter grbiciae* and *Geobacter metallireducens*	AF335182, L07834
50	CGGCTATTAACCGCACACATTT	*Geobacter pelophilus*	U96918
51	AGGTATTAGCTCTCAATCATTT	*Geobacter sulfurreducens*	U13928
52	GATTATTAGTCGTGCCACCTT	*Geothrix fermentans*	U41563
53	GGTTATTAACCCCCCAACCTT	*Geothrix fermentans*	AF034893
54	AGCTATTAACTCCCATGCTT	*Geothrix fermentans*	AF104277
55	GGCAGTTACTCCCCTTACCATT	*Geovibrio ferrireductans*	X95744
56	GGGTGTTATCCTGCAGGGGTTT	*Pelobacter acetylenicus*	X70955
57	GGCCTATTCGACCACGATAGTT	*Pelobacter carbinolicus*	U23141, X79413
58	GGGTATTAACCACAATACACTT	*Pelobacter propionicus*	X70954
59	GAATATTAACCCCTGGGTGTTT	*Pelobacter venetianus*	U41562
60	ATGTAAGGTATTAACTTACACC	*Shewanella algae* strain BrY	X81621
61	CTATAGGGTATTAACCTACAAC	*Shewanella amazonensis*	AF005248
62	TAAGCAGTTATTAACTACCTAC	*Shewanella hanedai*	AF187080
63	CAACAGCGTATTAAGCTGCTAC	*Shewanella massilia*	AJ006084
64	TAATAACGTATTAAGTTATTAC	*Shewanella putrefaciens*	U91549
65	ATAAGGCGTATTAAACCACACC	*Shewanella putrefaciens* ACAM-574	AF006670

Continued on facing page

TABLE 1—*Continued*

Probe	Sequence (5′–3′)	Target(s)	GenBank accession no.
66	ATGAGCCGTATTAAGACTCACC	*Shewanella oneidensis* SP-32 and SP-7	AF039056, AF039054
67	CAATGTGCTATTAACACATCAC	*Shewanella* sp. isolate Bok-1-30-2	AJ288152
68	TAATAGTTTATTAAACTATTACC	*Shewanella* sp. strain SIB1	AB039737
69	ATAAGCCGTATTAAGACTTACC	*Shewanella* sp. strain MR-7	AF005253
Nonsense probes[c]	dN_{20}		
	GATGATGATGATGATGATGA		
Chaperone/helper probes[d]			
1	ACGGGCGGTGTGTAC		
2	CTCCNCCGCTTGTGCGGG		
3	GCCGCCAGCGTTC		
4	TCTCAGTNCCANTGTGGC		
5	CGNTCGACTTGCATGT		
6	GTATTACCGCGGCTGC		
7	GAGCTTTACGACCCCG		
8	GAGGTTTACGATCCG		

[a] All capture probes were synthesized with a 5′ biotin. The two nonsense probes were used to correct for background, as described in Materials and Methods.
[b] The *Geobacter* group-level probe targets the 825 region of the 16S rRNA molecule and does not have a corresponding proximal chaperone.
[c] The two nonsense probes (synthesized with a 5′ biotin) were used to correct for background during the analysis of sediment samples, as described in Materials and Methods.
[d] Chaperone probes 7 and 8 specifically target the 420 region of metal- and sulfate-reducing bacteria, whereas chaperone probes 1 to 6 target secondary and tertiary structures elsewhere in the intact rRNA.

temperature. The RNA was collected by centrifugation, washed with 75% ethanol, dried under a vacuum, and reconstituted with 500 μl of lysis buffer.

Silica particles (catalog no. S-5631; Sigma Chemical) were prepared essentially as described elsewhere (21). A spin filter unit (Q-Biogene, Irvine, CA) was prewetted with 100 μl DMPC-treated water by centrifugation for 30 s at 14,000 × *g*. The filter was then placed into a clean catch tube, and an appropriate volume of suspended silica was added to the spin filter unit to obtain a final packed bed containing 40 μl of silica. The 40 μl of silica in the packed bed was then removed from the spin filter unit, placed into a fresh microcentrifuge tube, collected by low-speed centrifugation for 30 s, and washed three times with 500 μl DMPC-treated water and once with lysis buffer. The washed silica was then resuspended by gentle pipetting in 500 μl of RNA extract, which represented 5 g equivalents of Old Rifle sediment.

TABLE 2. Bacterial isolates used for the bead array validation study

Organism	Source[a]
Bacillus atrophaeus (formerly *Bacillus globigii*)	ATCC 9372
Shewanella putrefaciens	ATCC 8073
Shewanella amazonensis	ATCC 700329
Shewanella putrefaciens CN-32	SMCC 567W
Shewanella oneidensis MR1	ATCC 700550
Desulfomusa bakii	University of Massachusetts
Desulfomonas acetoxidans	University of Massachusetts
Malonomonas rubra	University of Massachusetts
Geobacter chapellei	University of Massachusetts
Geobacter bemidjiensis	University of Massachusetts
Geobacter metallireducens	University of Massachusetts
Geobacter pelophilus	OCM 797
Geobacter sulfurreducens	University of Massachusetts
Geobacter bremensis	OCM 796
Desulfovibrio desulfuricans G20[b]	ATCC BAA-1058
Desulfovibrio vulgaris	OCM 547
Desulfotomaculum putei TH-11	OCM 459
Desulfotomaculum acetoxidans	OCM 656
Sulfate-reducing bacterium strain CN-121	OCM 622
Desulfotomaculum strain YM-SRM	OCM 652
Desulfotomaculum orientis	OCM 655
Iron-reducing bacterium IR-125	OCM 722
Iron-reducing bacterium IR-231	OCM 729
Desulfomicrobium strain AYPOGEIU	OCM 750

[a] ATCC, American Type Culture Collection, Manassas, VA; OCM, Oregon Collection of Methanogens, maintained by David Boone at Portland State University, Portland, OR. University of Massachusetts isolates were a generous gift from the laboratory of Derek Lovley (Amherst, MA).
[b] *D. desulfuricans* G20 was originally isolated by Judy Wall and colleagues and has been deposited in the ATCC as isolate BAA-1058; it may be renamed *Desulfovibrio africanus* (J. Wall, personal communication).

The RNA-silica suspension was gently rocked for 2 min and centrifuged at 5,500 × *g* for 1 min, and the supernatant was removed and discarded. Another 500 μl of lysis buffer was gently mixed with the silica particles by gentle pipetting and then centrifuged again as described above, and the supernatant was discarded. The silica containing bound RNA was washed twice with 500 μl of 75% ethanol and twice with 500 μl of 100% ethanol and then dried in a heat block for 3 min at 90°C with the cap open. Immobilized and purified RNA was fragmented by adding 30 μl of 1× fragmentation buffer (5) to the dried silica, mixing the preparation with gentle pipette action, and incubating the preparation for 30 min at 90°C. The reaction was stopped by placing the tube on ice, and RNA was reprecipitated onto the silica with 0.1 volume of 3 M sodium acetate (pH 5) and 2 volumes of cold 100% ethanol for 2 min. The silica was collected by centrifugation at 5,500 × *g* as described above, and the supernatant was discarded. Silica particles were then washed with 150 μl of cold 100% ethanol and twice with 500 μl of 75% cold ethanol, with the supernatant discarded after each wash, and then dried in a heat block as described above.

Immobilized, fragmented RNA was then reconstituted in 30 μl of labeling buffer (Ulysis nucleic acid labeling kit; Molecular Probes, Eugene, OR) by using gentle pipetting. A capped tube was placed in a heat block at 90°C for 3 min to disrupt secondary structures in the RNA. Ten microliters of Alexa 532 (in dimethylformamide) was then mixed with the silica-labeling buffer cocktail while the tube was still in the heat block. After 10 min of incubation at 90°C, the labeling reaction was quenched by immersing the tube in an ice bath. For all manipulations involving the Alexa Fluor, special care was taken to minimize exposure of the sample to light. Labeled RNA was reprecipitated onto the silica for 2 min as described above. Free Alexa was removed from the silica-RNA by successive washes in 150 μl of 100% ethanol and 300 μl of 75% ethanol, and the supernatant was discarded after each wash. Silica particles containing fragmented and labeled RNA were then suspended in 300 μl of 75% ethanol and transferred into a fresh, prewetted spin filter and centrifuged at 14,000 × *g* for 3 min. The catch tube was replaced, and the spin filter unit containing the silica was placed in a 90°C heat block for 2 min with the cap open. The unit was removed from the heat block, 40 μl of prewarmed (70°C) 10 mM sodium bicarbonate (pH 8.5) was added, and the unit was incubated at 70°C for 2 min and centrifuged as described above to elute the fragmented and labeled RNA into a catch tube. The

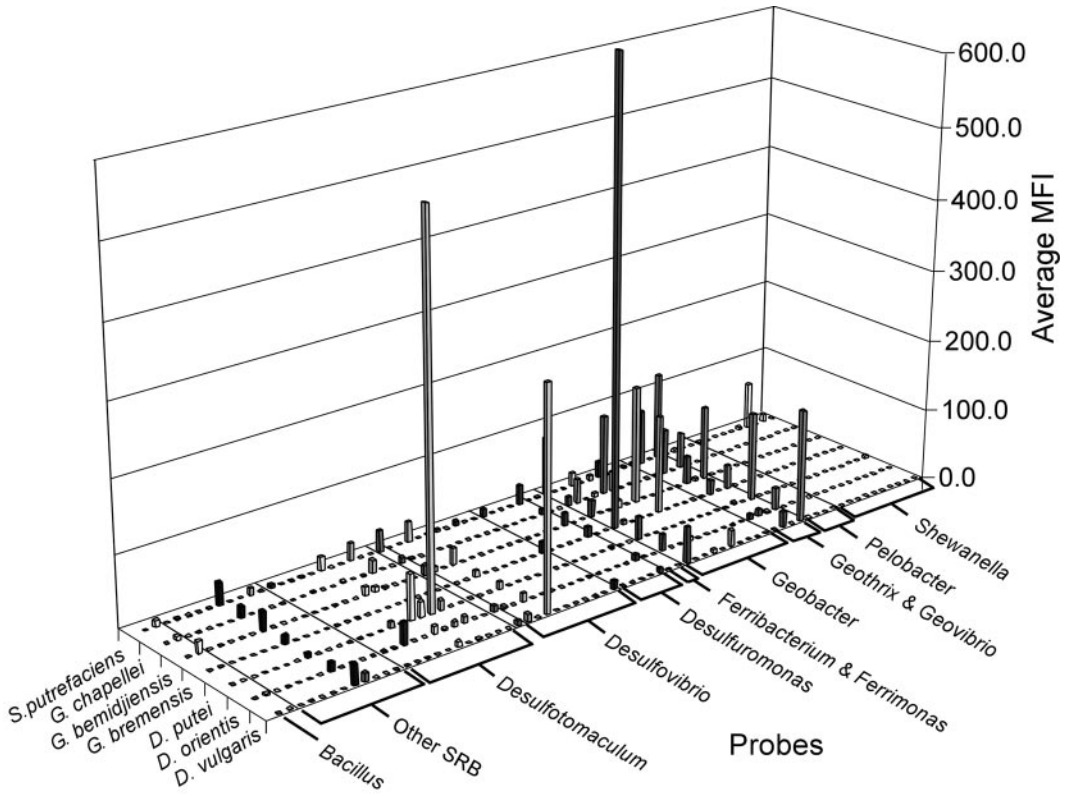

FIG. 2. Background-corrected validation array results for 100 ng of fragmented and labeled rRNA. The average MFI was calculated from three replicate hybridizations. For clarity, probes are aligned (from Other SRB to *Shewanella*) in this and other figures starting with *Bacillus* probe 1 in Table 1 and ending with probe 68 for *Shewanella* MR7. Only a fraction of the isolates are shown for clarity and to illustrate the major points discussed in the text. SRB, sulfate-reducing bacteria.

silica elution procedure was repeated two more times with 20 μl of prewarmed sodium bicarbonate as described above. The resulting RNA fraction was desalted on a Micro Bio-Spin 30 chromatography column (Bio-Rad, Hercules, CA) with a buffer exchange with DMPC-treated water, as recommended by the manufacturer. The final RNA yield was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the total volume of purified, fragmented, and labeled RNA was reduced to ~14 μl by vacuum centrifugation.

The purified, fragmented, and labeled sediment RNA (5 g sediment equivalents) was amended with 1 μg sheared salmon sperm DNA (Sigma-Aldrich, St. Louis, MO), eight chaperone/helper probes (Table 1), and DMPC-treated water to obtain a final volume of 17 μl. Nucleic acids were denatured at 95°C for 5 min, and the solution was added directly to 33 μl of the suspension array (5,000 beads for each probe) in 1.5× hybridization buffer (3× SSC, 0.03% Tween 20; pH 5) to obtain a final concentration of each chaperone probe of 250 nM in 2× SSC-0.02% Tween 20 (pH 5) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The hybridization reaction mixture was incubated for 2 h at 45°C as described above for the validation studies. After hybridization, the beads were collected by low-speed centrifugation (2,200 × *g*) for 2.5 min, washed with 300 μl wash buffer (2× SSC, 0.02% Tween 20, 0.5% Sarkosyl; pH 7.0), and resuspended in 150 μl analysis buffer (1× SSC, 0.01% Tween 20, 0.25% Sarkosyl; pH 7.0). The bead suspensions were disaggregated by vortexing before samples were injected into the flow cytometer, and the samples were injected at a rate of 60 μl min^{-1} with the Luminex cytometer set to count 100 events per bead type. A calibration run was performed with CAL1 and CAL2 calibration microspheres each day according to the Luminex instructions. The flow cytometer was controlled from a Pentium-II PC, and all data analysis was performed with the LMAT software (version 1.7; Luminex).

Statistics. There are a number of "statistical" methods to visually display differences in microarray profiles, such as dendrograms, principal-component analysis, scatter plots, and correlation coefficients. On the other hand, we are not aware of a formal statistic to test the hypothesis of profile equivalence, where a robust test of profile equivalence requires true replication. Furthermore, profile equivalence may be defined by a digital pattern of on/off probe responses or may take into account individual probe signal intensities in the data vector, as we have described in detail previously (38). As discussed below, the fundamental statistical challenge that we faced in this study was the fact that microbial spatial heterogeneity within the sediment samples precluded replication in the conventional biological or statistical sense, and definition of the appropriate scale for sampling and averaging in the subsurface continues to be a subject of intense dialogue. For this reason, we used a simple paired *t* test (in MatLab) for comparing microarray profiles. Raw or normalized signal intensities for each probe were subtracted one profile from the other. The resulting values were then averaged over all probes, and tested under the null hypothesis that the average of $P_1 - P_2$ was 0. A chi-squared test of independence could also be utilized, as described previously (22). A paired *t* test (probe by probe) was also used to tentatively identify probe responses that contributed to differences in microarray profiles.

RESULTS

Validation study. The purpose of the validation study performed with pure isolates was to understand the nature and extent of cross-hybridization before the array was used to analyze Old Rifle sediment samples. As discussed elsewhere (6), it is certainly possible to design (in silico) perfectly specific probes (including those listed in Table 1) to target rRNAs, but the likelihood that any oligonucleotide probe will behave perfectly in vitro and in uncharacterized environmental backgrounds cannot be determined a priori for all possible targets and background conditions. Figure 2 shows that there was cross-hybridization to nontarget species despite previously optimized hybridization conditions that included RNA fragmen-

tation, proximal helper probes for disrupting the secondary and tertiary structure, tunable surface hybridization conditions, and elevated hybridization stringency (5, 7). Some of the cross-hybridization was expected, based in part on the number of mismatches between the helper probes and all possible targets interrogated by the array. For example, the 420 region helper probes used here had more than three nucleotide mismatches (for both helpers) with rRNAs of *Geothrix*, *Sulfurospirillum*, *Desulfuromusa*, and *Desulfitobacterium* targets (represented by eight array probes); indeed, the *Geothrix* species-specific probes were highly cross-reactive, regardless of the target RNA. All other target-helper combinations, however, had less than three mismatches for at least one of the two helper probes (not shown). Hence, the number of mismatched nucleotides in the helper probe sequence may not account for all of the cross-hybridization data (e.g., the single *Geovibrio* probe).

If one makes the simplistic assumption that an organism with more rRNA operons generates more rRNA transcripts per unit of total RNA than an organism with fewer rRNA operons generates, then differences in absolute signal intensity (background-corrected, average MFI) between probes and organisms may be partially (but not entirely) attributed to an rRNA copy number effect. For example, the *Desulfovibrio vulgaris* probe resulted in an average MFI of 308 for the *D. vulgaris* target RNA (five genome copies) (18), while the *Geobacter sulfurreducens* probe resulted in an average MFI of 164 (two genome copies) (24). On the other hand, it is also clear that not all probes hybridize with the same efficiency, even though all probes target the same region of the 16S rRNA molecule. The *Geobacter bremensis* probe, for example, had a background-corrected average MFI of 650 for the *G. bremensis* target RNA but an average MFI of 744 for the *Geobacter pelophilus* target that differed by 11 (of 22) nucleotides in the target region! Both values are 4 to 4.5 times higher than the *G. sulfurreducens* probe response and ~6 times higher than the *Geobacter chapellei* probe response, and *G. bremensis* and *G. pelophilus* do not contain 8 and 12 rRNA operons, respectively. Likewise, all of the validation study results were predicated on application of 100 ng of total fragmented and labeled RNA to the array; the finding that the *Geobacter* probes were so much more reactive than other perfectly matched probe-target combinations with 100 ng of input RNA, therefore, cannot be attributed to rRNA copy number alone (e.g., compare the *G. bremensis* probe response to the response to any of the *Shewanella* control targets and probes).

These results are consistent with and similar to conclusions reached by Guschin et al. (15), which indicated that differences in the efficiencies of probe hybridization to target rRNA (as measured by absolute signal intensity) are compensated for by adjusting the concentration of immobilized probe within a three-dimensional gel element to obtain equivalent signal intensities with equivalent target inputs. Thus, the basic observations from the validation bead array are not new. Nonetheless, we conclude that 16S rRNA probe cross-reactivity to rRNA targets on the tunable bead array surface cannot be predicted based on the primary sequence and that it is currently unjustifiable to use probe signal intensity as a means to quantify the absolute abundance of organism A relative to the abundance of organism B in environmental samples in the absence of complete validation with all possible targets and environmental backgrounds. Hence, the conservative approach to interpretation of microarray data was to restrict our analyses to the comparative and relative responses of probe A to probe A over the temporally and spatially correlated sediment samples instead of comparing signal intensities from probe A to signal intensities from probe B for the same samples in a quantifiable manner. The meaning that we ascribe to a probe name within the context of an uncharacterized environmental sample is discussed below.

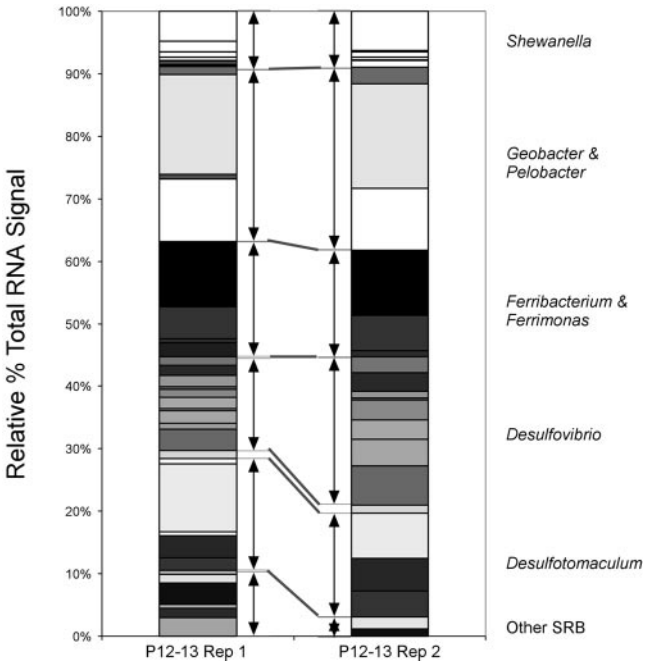

FIG. 3. Method-level reproducibility, deduced from replicate 5-g sediment aliquots from sample P12-13l. The background-corrected average MFI for individual probes was divided by the total MFI for the entire array to obtain a POT value. Individual probes are arranged from 1 (bottom) to 68 (top) consistent with Table 1; lines between the two bar graphs show the higher-order taxonomic bins that are consistent with classifications in Fig. 2 and are provided because individual probe values cannot be distinguished in gray scale and in the scaled image. SRB, sulfate-reducing bacteria.

Method-level reproducibility. All sediment samples were processed as 10 0.5-g replicates and were pooled prior to fragmentation, labeling, and bead array hybridization. It was not possible to separate the question of method-level reproducibility from spatial heterogeneity in a native environmental sample or sediment core. Nevertheless, we performed a preliminary analysis of the method-level reproducibility with one of the sediment samples in order to qualitatively assess the extent of spatial heterogeneity and method reproducibility before we proceeded with all other sediments. Replicate 5-g sediments from the 2003 P12 core (13-ft depth interval, closest to the injection gallery) (Fig. 1) were processed in parallel and hybridized to replicate bead arrays as described in Materials and Methods. The MFI for each probe was normalized to the total bead array signal MFI (in a manner similar to comparing operational taxonomic units within a clone library), as shown in Fig. 3. The total MFI for all probes for the replicates were

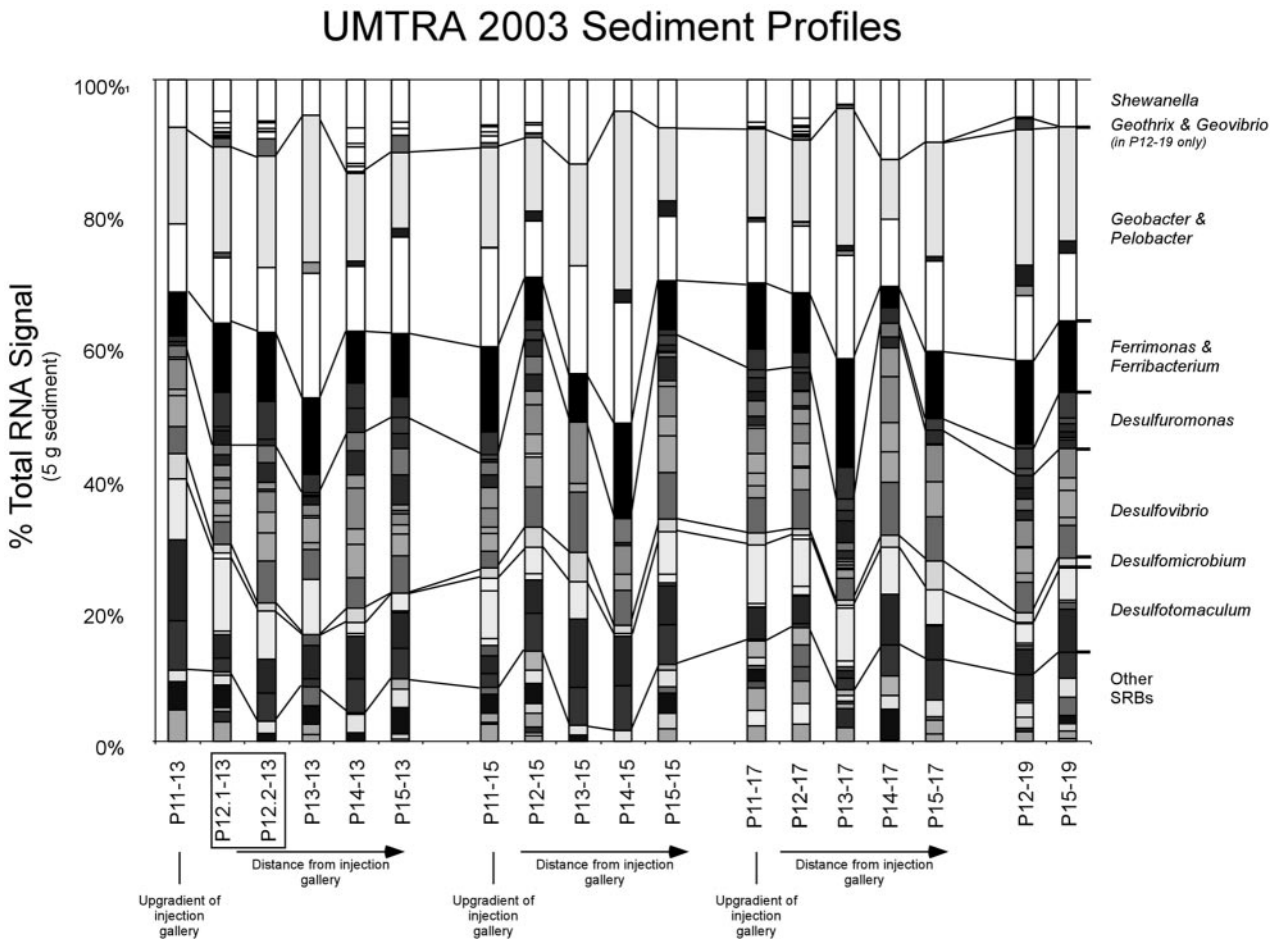

FIG. 4. Microbial community structure in 2003 sediment cores as a function of depth and distance down-gradient from the injection gallery, based on a POT calculation. The replicate P12-13 samples are replotted from Fig. 3. The numbers after the sample identifiers indicate depth (13, 15, 17, or 19 ft). SRBs, sulfate-reducing bacteria.

1,300 and 700, whereas the data in Fig. 2 indicate that individual signals up to 800 MFI did not saturate the beads. Based on the simplistic paired *t* test, the two profiles are statistically distinct at an α value of 0.05 with an R^2 value of 0.876. The paired *t* test for 68 probes revealed 22 significant differences between profiles at an α value of 0.05 and 13 differences at an α value of 0.01. The probes that contributed to the *t* test statistical difference included probes for *Desulfoarculus baarsii*, *Desulfobulbus elongatus*, *Desulfotomaculum* (four probes in the panel), *Desulfovibrio* (four probes in the panel), *Desulfuromonas chloroethenica* and *Desulfuromonas bakii*, *Ferribacterium limneticum*, *Geobacter hydrogenophilus* and *Geobacter pelophilus*, *Geothrix fermentans*, *Geovibrio ferrireductans*, and *Shewanella* (four probes in the panel). Whether the differences were due to spatial heterogeneity, the relative abundance of specific microorganisms in the sediment, or measurement error could not be determined absolutely with the methods used here. Based on the results of the validation study which are shown in Fig. 2, in which true biological replicates were available, however, we believe that the statistically significant differences in microarray profiles described here were due to spatial heterogeneity within the 5-g sediment samples and not to bead array measurement error. The data therefore indicate that it is important to evaluate a microarray profile over time and/or through space in order to identify higher-order trends in the data that reflect the underlying microbial community response. Nevertheless, the results provided sufficient confidence in method-level reproducibility that we performed a comparative analysis of sediment samples from the 2002 and 2003 field trials.

Community profiles with depth and down-gradient distance from the injection gallery. If we accept the conclusion that the protocols described here represent a reproducible method (from sample to answer), then we can begin evaluating microbial community response as a function of depth and distance down-gradient from the injection gallery. In this sense, the bead array signals are generally consistent with the results of previously described clone library analyses at the site, in that known genera of metal- and sulfate-reducing bacteria are present (3, 36) and the corresponding bead array probes for metal- and sulfate-reducing bacteria show significant signals over background (e.g., *Geobacter*, *Desulfotomaculum*, *Desulfovibrio*, and other sulfate-reducing bacteria). Similar bead array views of microbial community structure were generated for the 2002 and 2003 sediments, as shown in Fig. 4 for the 2003 sediment series and in Table 3 for all samples. In general, the

TABLE 3. Sediment sample statistics

Yr	Borehole[a]	Depth (ft)	Total RNA (μg)[b]	Base-to-dye ratio[c]	Total MFI	Shannon-Weaver diversity index[d]	Richness[e]	Evenness[f]
Background[g]	B01	14	0.4	16	993	2.55	30	0.75
		16	0.5	15	721	2.08	24	0.65
	B02	12	1.3	25	1,586	4.69	45	1.23
		19	1.0	17	666	2.16	35	0.61
	M03	11	1.9	30	2,138	5.89	47	1.53
		14	0.7	22	1,022	2.96	32	0.85
		18	0.3	10	526	1.99	34	0.56
	M08	12	0.8	22	970	3.38	44	0.89
		14	0.6	14	658	2.65	49	0.68
		19	0.5	17	715	2.11	28	0.63
	M13	12	2.0	33	2,300	6.12	50	1.56
		15	0.8	14	2,148	5.29	57	1.31
2002	P01	13	1.4	30	1,386	4.08	43	1.09
		15	2.3	32	1,110	3.65	47	0.95
		17	1.7	35	768	2.93	53	0.74
	P02	13	1.5	33	1,148	3.85	48	0.99
		15	5.8	42	3,100	7.37	49	1.89
	P03	13	2.5	37	1,789	5.10	46	1.33
		15	0.9	20	545	2.11	44	0.56
		17	1.0	46	153	0.76	36	0.21
	P04	13	4.2	41	2,076	5.52	43	1.47
		15	4.9	47	2,068	5.29	45	1.39
		17	0.6	18	323	1.28	26	0.39
	P05	13	3.5	38	2,104	5.48	49	1.41
		15	1.6	34	1,440	4.54	49	1.17
		17	1.7	43	466	1.91	48	0.49
	P06	13	2.5	53	1,508	4.69	51	1.19
		15	2.0	37	642	2.39	42	0.64
		17	2.0	37	72	ND[h]	ND	ND
2003	P11	13	0.3	11	493	1.66	19	0.57
		15	2.0	45	1,028	2.96	31	0.86
		17	0.3	10	536	1.99	35	0.56
	P12	13	1.9	32	1,570	4.08	38	1.12
		15	0.6	31	1,148	3.51	35	0.99
		17	1.1	19	676	2.39	38	0.66
	P13	13	0.5	12	424	1.43	22	0.46
		15	1.1	17	313	1.10	13	0.43
		17	1.3	41	944	2.60	33	0.74
	P14	13	1.5	55	390	1.53	28	0.46
		15	1.3	31	208	0.79	15	0.29
		17	ND	ND	388	1.46	20	0.49
	P15	13	ND	ND	391	1.53	27	0.46
		15	0.7	68	469	1.77	28	0.53
		17	ND	76	434	1.50	20	0.50

[a] See Fig. 1 for the locations of sediment cores.
[b] Amount of total RNA after sediment extraction and purification. The entire bolus was processed through the fragmentation and labeling protocol and was hybridized to the bead array as described in the text.
[c] Base-to-dye ratio in the labeled RNA, calculated by using Molecular Probes protocols and rounded to the nearest integer.
[d] The Shannon-Weaver diversity index (H) was calculated as follows: $H = -\Sigma p_i[\ln(p_i)]$, where p_i is the proportion of the total array MFI for each probe (i), as shown in Table 1.
[e] Richness was defined as the absolute number of background-corrected probe responses (for 68 probes listed in Table 1) that were greater than zero.
[f] Evenness (E) was calculated as follows: $E = H/[\ln(S)]$, where H is the Shannon-Weaver diversity index and S is richness.
[g] Background samples collected prior to the 2002 and 2003 acetate amendments.
[h] ND, not determined.

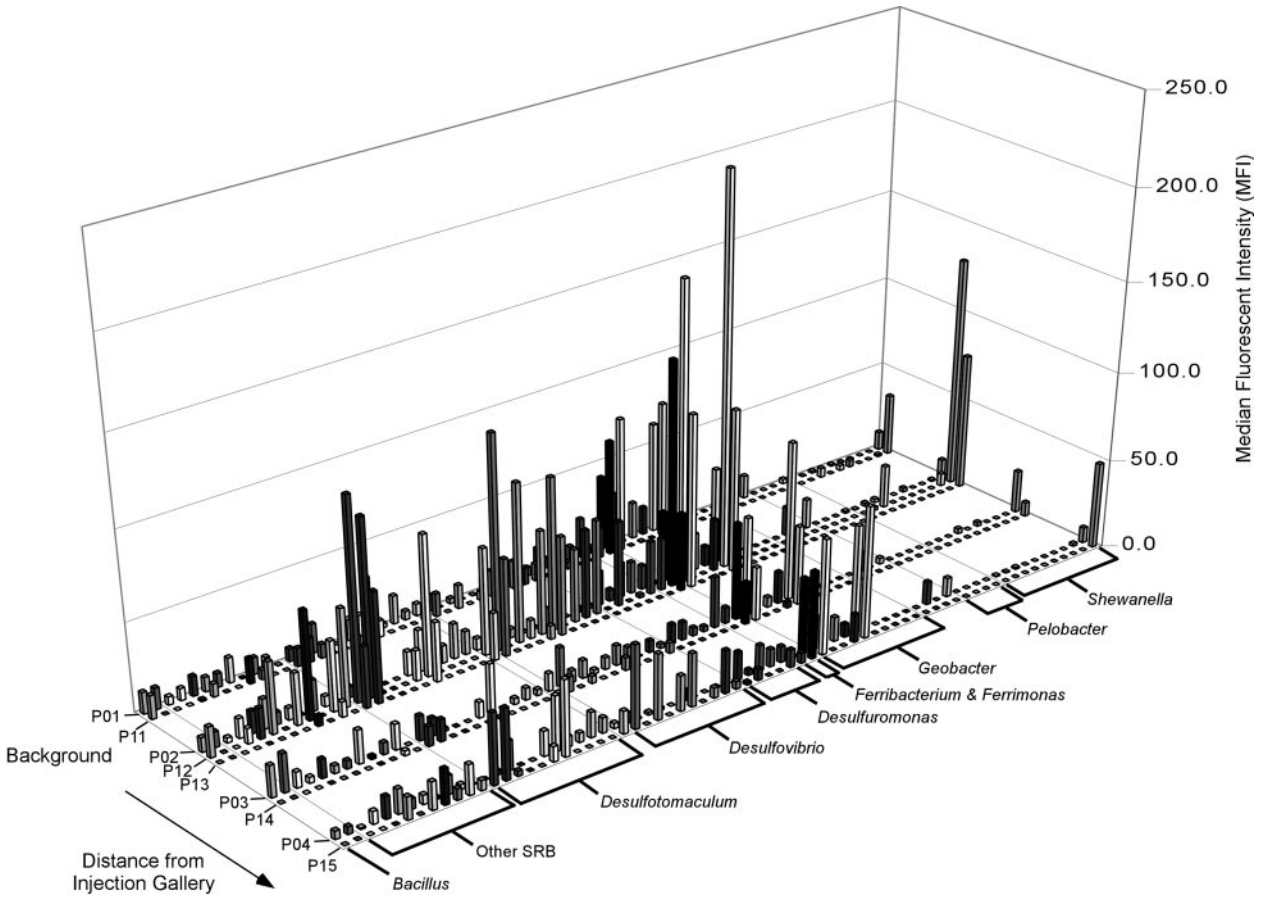

FIG. 5. 2002 and 2003 microbial community structure, with individual background-corrected probe intensities normalized to the total amount of purified, fragmented, and labeled RNA applied to the bead array (all from 5 g of sediment). Samples whose designations begin with P0 are from the 2002 treatment, and samples whose designations begin with P1 are from the 2003 treatment. Only data from the 15-ft depth interval are shown. SRB, sulfate-reducing bacteria.

profiles for both 2002 and 2003 sediments show fine-scale differences with depth and/or down-gradient distance from the injection gallery, and there are few drastic or abrupt changes in the bead array profiles according to group-level bins or individual probe responses. This is in contrast to the results of clone library analyses (36), in which sharp microbiological gradients were observed.

We were moderately surprised by these data and the apparent stability of community profiles across time (years), in space, and with distance down-gradient from the injection gallery, since the total amount of recovered RNA, the estimated biomass, and the total bead array signal (total MFI) varied substantially with depth and down-gradient distance (Table 3). Despite the general observation that more total RNA applied to the array generated a higher total MFI signal (Table 3), there was not always a one-to-one correspondence between total RNA recovered and total MFI. For example, 2.0 μg of total RNA from 2002 sample P06-15 (closest to the injection gallery) resulted in a total MFI of 652, whereas 1.4 μg of total RNA from 2002 sample P05-15 (farthest from the injection gallery) resulted in a total MFI of 1,440. While one could argue that the different total MFI for the two sediments were due to method-level variability, data in Fig. 3 and the fact that we counted the total microarray signal (instead of individual probe responses) suggested that there was a difference in the relative abundances of metal- and sulfate-reducing bacteria between the two sediments and/or a difference in the total metal- and sulfate-reducing populations extracted from the different 5-g sediments. Put another way, total RNA recovery may be considered a proxy for the size and activity of the entire microbial community in the sample, whereas total MFI may be considered a proxy for the size and activity of the metal- and sulfate-reducing subpopulation that is interrogated by the array. However, the question of or uncertainty about microbial spatial heterogeneity was still not resolved by considering RNA recovery and total MFI on the array, since the 100- to 500-g core samples were not fully homogenized before bead array analysis. Regardless, we suggest that the percentage-of-total (POT) view of the bead array response (Fig. 4) may be missing important information about the underlying community structure or dynamics and that alternative views of the bead array data might provide more meaningful ecological information.

Normalized view. Using the argument described above, we normalized probe intensity values based on the total RNA recovered from each 5-g sample, which resulted in the community profiles shown in Fig. 5. Whereas the data in Fig. 4 and

Table 3 (Table 3 shows raw intensity data) reveal that there was relatively little difference in (fine-scale or gross) community structure with depth, down-gradient distance, or treatment regimen, Fig. 5 clearly shows that there was a general increase in normalized probe signal intensities near the injection gallery and relative to the up-gradient sediment background, which tapered off to near-background levels with increased down-gradient distance from the injection gallery. Similar views were obtained from other depth intervals (13 and 17 ft). All possible pairwise *t* tests for all samples listed in Table 3 suggested that the only microarray profiles that were statistically indistinguishable from each other at an α value of 0.05 were pairs P04-13/P01-13, P04-13/P03-13, P05-15/P06-13, P06-17/P02-13, and P06-17/P06-15 for the 2002 sediments and pairs P15-13/P14-13, P13-15/P12-13, P14-17/P12-17, and P13-19/P13-17 for the 2003 sediments. There were 13 pairs of microarray profiles that were indistinguishable in the 2002 and 2003 sediments. The normalized view of the microbial community response to acetate injection is also consistent with expectations and prior observations at the site, in that a majority of in situ microbial activity occurred near the injection gallery and the normalized bead array response (which was a direct measure of rRNA or general microbial activity) was consistent with previous observations and reports (3).

One interesting observation from the bead array was the relatively strong rRNA signal for the *Shewanella* MR7 probe, since *Shewanella* signatures or clones were not identified at the site in previous analyses. However, we are guarded about the ecological meaning of this signal in light of the validation study (Fig. 2), in which control *Shewanella* rRNA targets generated little (absolute) signal on the bead array. Verification that the MR7 probe response was due to a *Shewanella*-like rRNA would best be accomplished by performing follow-up studies, perhaps using *Shewanella*-specific 16S rRNA PCR amplification. Subtle differences in fine-scale community structure within the major classes of metal- and sulfate-reducing bacteria were also noticeable, although (as discussed above) it was difficult to determine if these differences (e.g., differences between treatments or differences with down-gradient distance from the injection gallery) were due to microbial spatial heterogeneity and sample size or represented differences in the microbial community response to the acetate treatments. Also, while it is tempting to conclude that the exceptional *Geobacter* signals correlated with abundance and/or activity in the sediment (as opposed to clear abundance and activity in the aquifer, as reported previously [3]), such a conclusion is not warranted at this time given the exceptional signal and responsiveness of *Geobacter* probes observed during the validation study (Fig. 2) (see above).

Relative community response. While the normalized probe intensities are more informative than absolute probe signal intensities and show individual and community-wide responses consistent with expectations and previous work at the site, the magnitude of a normalized signal response does not directly address the question of probe, microbial, or community responsiveness to acetate treatment. Indeed, both the 2002 and 2003 normalized profiles (Fig. 5) are remarkably consistent even though acetate injections were modified for the 2003 field season in order to satisfy the sulfate-reducing bacteria near the injection gallery and stimulate downstream metal-reducing populations and activity. Therefore, in order to more clearly identify responsive portions of the metal- and sulfate-reducing subpopulation, we took the normalized signal intensity for each probe and calculated a fold change in the normalized probe intensity for probe A compared to probe A in each of the sediments, using the background sediment samples (P01 and P11) immediately up-gradient of the injection gallery as the environmental reference point. Results obtained in 2002 and 2003 for the 15-ft depth interval are shown in Fig. 6A and B, respectively. A number of interesting observations and conclusions arose from this analysis, not only for the interpretation of microbial community response but also for the underlying bead array technology. For example, the increased acetate concentration in 2003 appeared to have specifically stimulated the sulfate reducers compared to their lack of down-gradient responsiveness during the 2002 campaign, as hoped and anticipated by the team of ecologists and bioremediation engineers. Of particular interest, however, was the nonresponsiveness of the *Geobacter*, *Ferrimonas*, and *Ferribacterium* probes relative to the normalized background signal, given the relatively large absolute (Fig. 4) and normalized (Fig. 5) signal intensities. Likewise, the single *Geobacter* SBD-1 probe, with which there was a 30-fold increase in the normalized signal intensity during the 2003 campaign (Fig. 6B, sample P12-15), was actually one of the weakest responding probes as determined by any other calculation or analysis (indeed, it was not detectable in the background and a majority of the sediment samples [Fig. 4 and 5]). Also of interest was the finding that the *Desulfotomaculum* probes suggested that there was a relatively strong response in the 2003 treatment compared to the 2002 treatment (Fig. 5), which qualitatively correlated with the fold change data shown in Fig. 6B for the *Desulfotomaculum acetoxidans*, *Desulfotomaculum reducens*, *Desulfotomaculum nigrificans*, and *Desulfotomaculum alkaliphilum* probes. However, the finding that the *Geobacter* probes with the highest absolute or normalized signal intensities showed very little response in the spatially or temporally correlated samples was a further indication that signal intensity (absolute or normalized) is not necessarily an accurate predictor of in situ microbial abundance or activity as the bead array assay is currently configured, so definitive conclusions regarding microbial activity in sediments require follow-up analysis with other techniques. Likewise, responsive probes can and do arise from this type of data presentation simply because their levels are below the levels of detection in the reference sample, which forces us to question (ecologically and practically) what it means for a microorganism or microbial community to be responsive to treatment when molecular techniques are used to measure in situ community dynamics.

Principal-component analysis (Fig. 7) of the normalized signal intensity data in Fig. 5 easily distinguished microarray profiles for the 2002 and 2003 acetate treatments, and in particular, sediment core P12-15 was clearly distinct from the cores from the other boreholes down-gradient from the injection gallery in 2003. Four of the principal components separating 2002 sediments from 2003 sediments were probes targeting *Desulfotomaculum*, whereas four of the principal components separating P12-15 from the other 2003 sediments were probes targeting the *Desulfovibrio* species. The

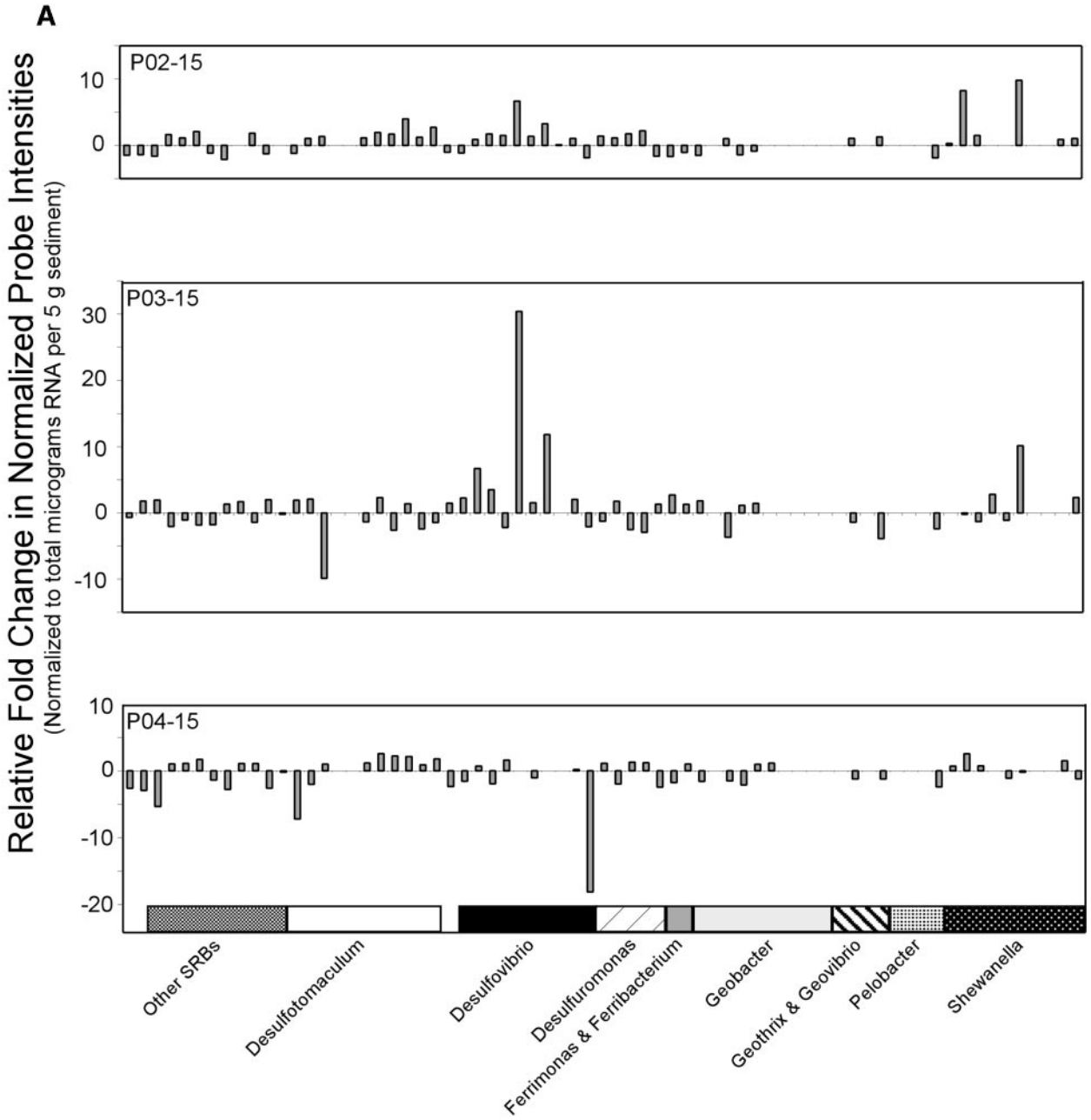

FIG. 6. Relative changes (n = fold) in background-corrected, normalized (to total RNA from 5 g of sediment) probe intensities in the 15-ft depth interval and with increasing distance from the injection gallery (see Fig. 1). Probes whose levels were below the detection level were assigned an average MFI of 1 for the purpose of calculating a fold change. (A) 2002 sediments relative to the P01 background. (B) 2003 sediments relative to the P11 background. SRBs, sulfate-reducing bacteria.

sharp separation of P12-15 from the other sediments may have been due to uneven distribution of acetate during the 2003 injection, as previously hypothesized (36). In either case, the principal components were heavily weighted by probes for the sulfate-reducing genera and not probes for the *Geobacteraceae*. These observations may support the hypothesis that there is differential distribution or activity of *Geobacteraceae* between sediment and groundwater at the UMTRA site (3, 36).

DISCUSSION

Utility of microarrays in an environmental context. The results of this study raise some important analytical questions for the environmental microbiology user community, as partially discussed elsewhere (6). Given the observed cross-reactivity on the arrays and the apparently unpredictable nature of 16S rRNA-targeted probes, the most pressing questions are whether the array data are believable and if they can be used

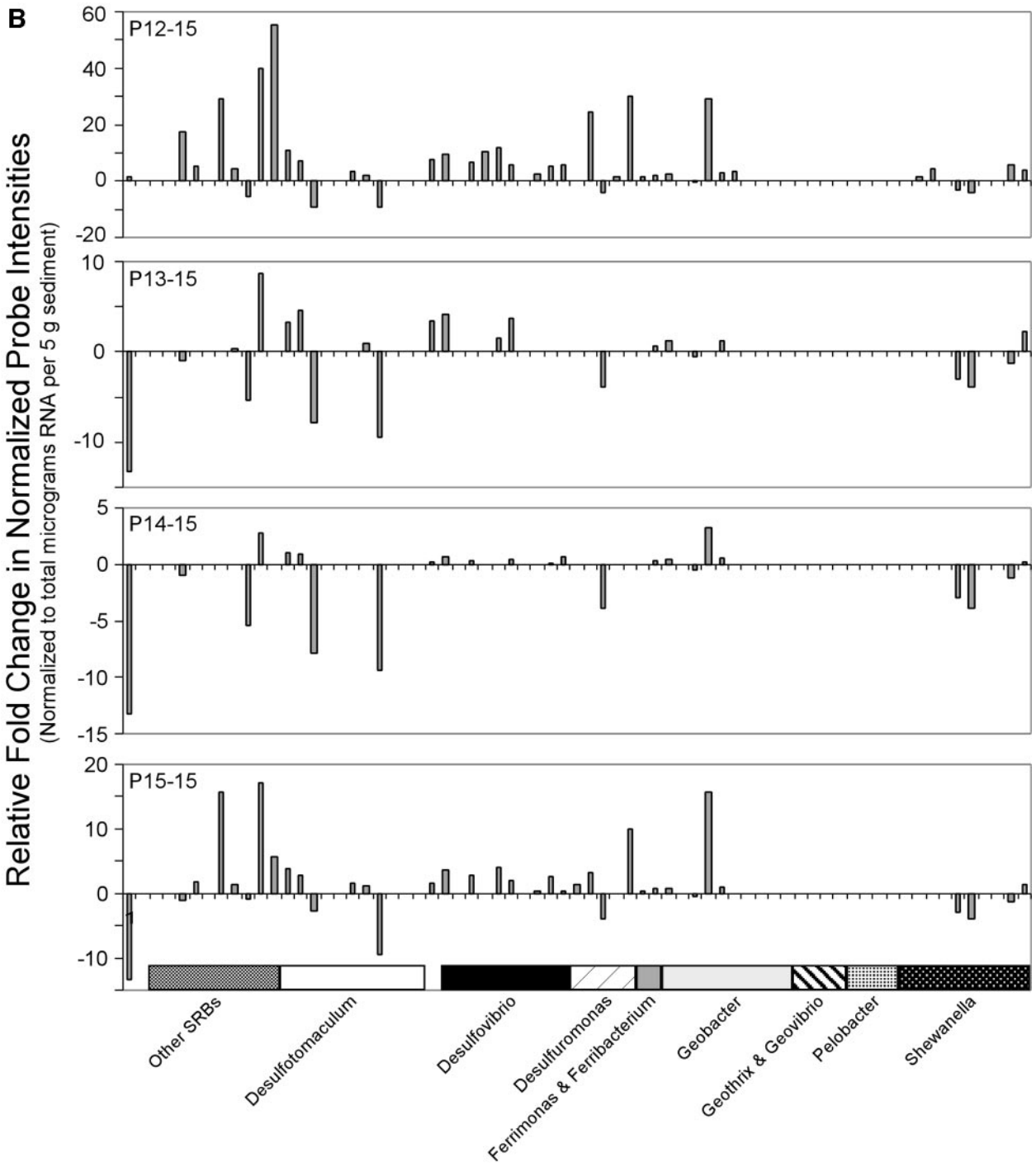

FIG. 6—*Continued.*

to draw microbiological and ecological conclusions at the site. There are two lines of argument (analytical and empirical) that buoy our enthusiasm for continued use and development of rRNA-targeted microarrays for environmental applications and lead to the conclusion that the data described above are indeed believable and provide meaningful information concerning the underlying microbial community and efficacy of acetate injection.

Analytically (and based on the sheer number of peer-reviewed publications), it is accepted that PCR-generated 16S rRNA clone libraries and denaturing gel electrophoresis and terminal restriction fragment length polymorphism profiles are ecologically informative, and many conclusions regarding in situ microbial abundance are predicated solely on a 16S rRNA clone library and percentage-of-total calculations. In this context, the array data presented here are ecologically informative

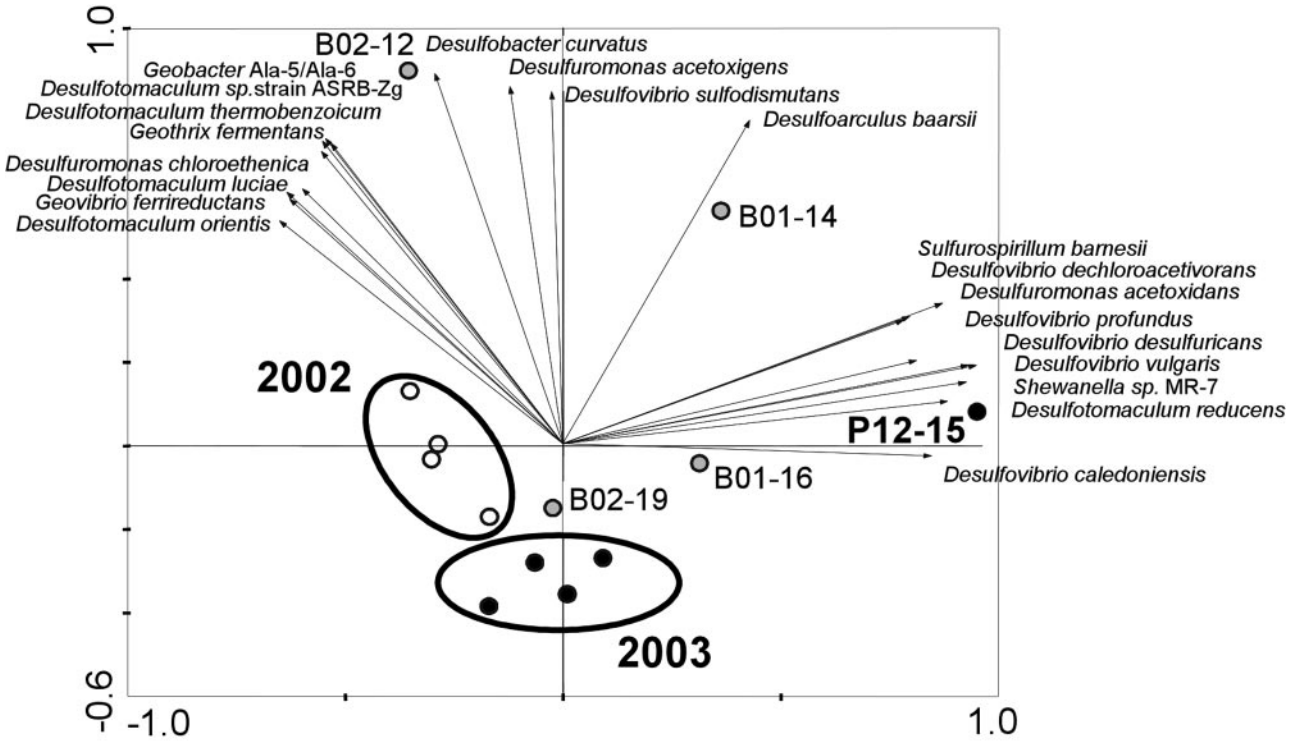

FIG. 7. Principal-component analysis for the 15-ft depth interval (normalized data from Fig. 5). The principal components shown account for 52% of the variation in the microarray data. Redundancy analysis of the binary data (presence or absence of specific probes) also showed that there was a significant difference between years ($P = 0.002$; not shown).

almost without question, because (i) multiplex, nonquantitative PCR was omitted from the bead array process defined here; (ii) we analyzed rRNA directly as a measure of generic microbial activity; (iii) all microarray capture and helper probes targeted the same region of the 16S rRNA molecule; and (iv) the method was successfully used with ecologically and environmentally relevant cell densities.

If the analytical and method-level argument is not convincing, then there are several empirical lines of evidence which support our conclusion and (measured) optimism for direct microarray analysis of 16S rRNA. First, we observed fine-scale differences in the microbial community structure with depth and distance down-gradient from the injection gallery, indicating that the 16S rRNA-targeted probes measured real differences in the rRNA composition between sediments. Second, the relative quiescence for a majority of the *Shewanella* probes (except probe MR7) indicated that the bead array and probe responses were not (biologically) random and exhibited some level of species or group specificity, since *Shewanella* has not been identified in any other sample or (microbiological and molecular) analysis of the site. Third, probes that were generally cross-reactive in the validation array regardless of the target RNA (i.e., *Geothrix* probes) or the probes that generated relatively strong absolute signal intensities regardless of the sediment (i.e., *Ferrimonas* and *Ferribacterium* probes) were not responsive in the fold change analysis of the data, indicating that the corresponding in situ organisms were largely unaffected by the acetate treatment. Finally, the probes that were responsive on the bead array are consistent with site chemistry and previously published data, including expected responses with the genera *Geobacter* and *Desulfotomaculum* and other sulfate-reducing genera.

While we conclude that the bead array data are believable and ecologically meaningful, we are purposefully measured in the ecological conclusions drawn from the bead array analysis (see below), and we urge caution especially with respect to the magnitude (absolute or relative) of the probe response and the name assigned to each of the 16S rRNA-targeted microarray probes. For example, evidence from the validation array (Fig. 2) and the normalized, relative change in the individual probe response (Fig. 6) indicated that additional process-level controls and/or fundamental studies of bead array behavior are required in order to accurately correlate probe signal intensity with absolute rRNA abundance in the environment, regardless of the measurement precision or reproducibility on the array itself. The extent to which these conclusions can be extended to other array substrates and/or other types of probes (e.g., 40- to 70-mer oligonucleotides or gene fragments) is not clear and cannot be deduced from this study, but they at least raise some important analytical questions that should be addressed before microarray platforms are used wholesale to draw microbiological conclusions based on uncharacterized environmental samples. Likewise, the data in Fig. 4 to 6 should engender caution in drawing ecological conclusions based on a POT analysis; that is, even though *Geobacter* and *Ferrimonas* probes were exceptionally strong in the POT analyses (Fig. 2 and 3), their relative responses for temporally and spatially correlated samples and with respect to the environmental background were

quite weak compared to the responses for other segments of the community measured (Fig. 6) and principal components (Fig. 7). The question that arises, then, is, which view of the microbial community (based on Fig. 4 to 7) is most meaningful for the ecologist or the bioremediation engineer?

Meaning of probe names in uncharacterized sample backgrounds. Exceptional effort and resources are expended on the a priori design of unique, species- or gene-specific probes with no known homology to any other sequence in public databases, so that the resulting nucleic acid data (whether from arrays, PCR, or related technologies) can be assigned to the cognate species after analysis of an uncharacterized sample. The probe design enterprise and goal are indeed necessary and virtuous, and 30 years of general nucleic acid technology experience with test tube and membrane hybridization (including dot blots, Southern blots, and various PCR methods) suggest that in silico design leads to expected probe behavior when the probes are translated into a new measurement method or platform. On the other hand, the results presented here and concerns over microarray cross-hybridization expressed elsewhere (13, 16, 17, 26, 40) force us to question the basic molecular assumption that unique probes behave uniquely and predictably on microarrays. Because we cannot define the environmental background a priori, statistical techniques and array designs predicated on measuring the signal intensity of a perfectly matched probe relative to an imperfect match cannot identify or militate against false positives due to cross-hybridization (because we cannot define a mismatch a priori in uncharacterized samples). On the other hand, the results presented here may simply indicate that direct rRNA detection and analysis with 20-mer oligonucleotide probes present some especially unique biophysical problems and behavior compared to other microarray platforms and assay formats. In any case, the analytical dilemma posed by false-positive hybridizations and uncharacterized sample backgrounds is that we cannot a priori assume that validation studies and efforts to achieve optimized hybridization conditions will translate directly to an environmental sample. The ecological dilemma is that we cannot necessarily assume that positive hybridization signals for specific probes (whether designed at the strain, species, genus, or higher level) directly measure the intended and desired target nucleic acids. How, then, can we draw any ecological conclusions from the bead array data presented here?

We believe that correlated samples are invaluable for making sense of microarray data. In the absence of correlated samples, questions concerning cross-hybridization (e.g., cross-hybridization on expression profiling arrays) can be addressed by quantitative PCR (e.g., to validate the up- or down-regulation of interesting transcripts), a verification step that can certainly be applied to environmental samples when the name of the detected or responsive organism is especially important to the end user. At the same time, the specific name of an organism or hybridized target RNA may not be required in order to draw ecological or bioremediation conclusions from uncharacterized samples; that is, rather than interpreting individual probe responses as absolute, precise indicators of the cognate gene or organism, we can instead think of the entire array response as a signature profile for a class of organisms or a community-level response to treatment. The analytical principle is akin to a generic pattern recognition problem that is used with great success in the design and use of gas-phase chemical sensor arrays, in which individual polymers on the array are not absolutely specific for a specific chemical (2), and is actually embedded (although not articulated) in cross-species use of high-density expression profiling arrays (20). This view of microarray probes and methods of data interpretation is partially responsible for our decision to design and use multiple species-specific probes for each genus of interest rather than one or a few genus-level probes and led us to interpret the community response according to more-generic microbial classes (as binned in Fig. 2 to 6) rather than as individual names. This decision was further supported by a clone library analysis of the 2003 sediments, from which a number of metal and sulfate reducer 16S rRNA gene clones were recovered that differed from the type species (36), where preexisting sequences would otherwise be used to design genus- or species-specific microarray probes (39). The extent to which quantitative statistics and decision rules can be developed to empirically link probe and/or array responses to a defined microbial class in the environment is to be determined, but this development will likely require new (and as-yet-undeveloped) standards and references inserted into the analytical method and array.

From the premise described above, an affirmative decision or conclusion concerning the efficacy of increased acetate concentrations during the 2003 injections can be reasonably made in part because there is a temporally and spatially correlated sample set which can be analyzed for responsiveness in time and space (Fig. 6 and 7). In the absence of a correlated sample set or related data, we believe that none of the bead array data presented here is informative to the bioremediation engineer in and of itself, especially since the majority of the microarray profiles were classified as profiles that were statistically distinct from each other. Corollary geochemical, biochemical, molecular, and microbiological evidence (3, 8, 12, 28, 30, 36) also supports the interpretations of the array data provided here (and vice versa). A more sophisticated correlation of the bead array response with geochemical properties, perhaps involving neural networks (29), undoubtedly would provide more robust, statistical support for the efficacy and impact of the acetate concentration on microbial community dynamics, although such an analysis was beyond the scope of the present study. The extent to which geochemical, hydrological, and microarray data can be correlated within a time frame to influence bioremediation decisions in the field is also to be determined, although the value of integrating converging lines of evidence before a treatment decision is made is inherently obvious. The fact that a bead array community response can be generated within 1 day of receipt of a sediment sample engenders confidence that microarray technology will ultimately be useful in the field.

Conclusions. From the study described above, we conclude that direct interrogation of rRNA with tunable bead arrays and a proximal capture-helper probe strategy can provide insight into microbial community structure and dynamics in low-biomass, subsurface sediments undergoing bioremediation. We suggest that making sense of the bead array data is best accomplished within the context of spatially and/or temporally correlated sample series and supporting lines of evidence, where the microarray metric of importance to the bioremediation engineer is probe responsiveness to a reference sample. In

the absence of complete validation with all possible targets and environmental backgrounds, we concluded that it is currently unjustifiable to use probe signal intensity (or, by extension, signal-to-noise ratios) as a means to quantify the absolute abundance of rRNA (let alone a microbe) in the environment or as a means to draw ecological conclusions about the abundance of organism A relative to the abundance of organism B in the environment. Despite these current limitations of the underlying bead array approach, the fact that the bead array data make sense at all is encouraging and suggests that continued development of field-deployable microarray systems and analysis techniques should provide timely, meaningful, and actionable information to bioremediation engineers, regional stakeholders, government regulators, and policy makers faced with site cleanup and long-term stewardship decisions.

ACKNOWLEDGMENTS

This work was supported by the Natural and Accelerated Bioremediation Research Program, Biological and Environmental Research, Office of Science, U.S. Department of Energy. Argonne National Laboratory is operated for the U.S. Department of Energy by the University of Chicago under contract W-31-109-ENG-38. Pacific Northwest National Laboratory is operated by Battelle for the U.S. Department of Energy under contract DE-AC06-76RL01830.

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