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Effect of Nitrate Injection on the Microbial Community in an Oil Field as Monitored by Reverse Sample Genome Probing

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The reverse sample genome probe (RSGP) method, developed for monitoring the microbial community in oil fields with a moderate subsurface temperature, has been improved by (i) isolation of a variety of heterotrophic bacteria and inclusion of their genomes on the oil field master filter and (ii) use of phosphorimaging technology for the rapid quantitation of hybridization signals. The new master filter contains the genomes of 30 sulfate-reducing, 1 sulfide-oxidizing, and 16 heterotrophic bacteria. Most have been identified by partial 16S rRNA sequencing. Use of improved RSGP in monitoring the effect of nitrate injection in an oil field indicated that the sulfide-oxidizing, nitrate-reducing isolate CVO (a *Campylobacter* sp.) becomes the dominant community component immediately after injection. No significant enhancement of other community members, including the sulfate-reducing bacteria, was observed. The elevated level of CVO decayed at most sampling sites within 30 days after nitrate injection was terminated. Chemical analyses indicated a corresponding decrease and subsequent increase in sulfide concentrations. Thus, transient injection of a higher potential electron acceptor into an anaerobic subsurface system can have desirable effects (i.e., reduction of sulfide levels) without a permanent adverse influence on the resident microbial community.

Oil fields at moderate subsurface depth and, consequently, with a moderate resident temperature harbor a complex microbial community that is being characterized in increasing detail (22, 27, 30). The metabolic potential of this community is characterized by an abundance of electron donors (aliphatic and aromatic hydrocarbons) but a shortage of electron acceptors (in order of increasing redox potential, carbon dioxide, sulfur and sulfate, ferric ions, nitrate, and oxygen). Stimulation and direction of the metabolism of this resident community are desirable for microbially enhanced oil recovery and to prevent the formation of excessive levels of sulfide (reservoir souring) by the action of sulfate-reducing bacteria (SRB), which has corrosive effects (7, 10). Both of these positive effects can be achieved by injection of nitrate, i.e., nitrate can serve as an alternate higher-potential electron acceptor (compared to sulfur or sulfate) and thus can stimulate the metabolic activity of the oil field microbial community. This can lead to increased oil recovery through microbial production of gas, emulsification of biopolymers, or the blockage of nonproductive subsurface channels (3). In addition, sulfide levels can be decreased through the action of bacteria that use sulfide as electron donor for nitrate reduction (12, 16, 26). However, some SRB are known to be able to use nitrate as well as sulfate as electron acceptors (18, 25, 32), and nitrate reduction coupled to sulfide oxidation may raise sulfate and sulfur levels. Thus, the increase in metabolic activity of the microbial population may be due in part to an unwanted increase in numbers and activity of SRB, for which reason it is important to document the shifts in microbial population that result from nitrate addition. The reverse sample genome probe (RSGP) method can track a large number of culturable bacteria simultaneously (23, 28, 30, 31), and the results of its application to monitoring the dynamics of an oil field microbial community following nitrate injection are presented here.

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MATERIALS AND METHODS

Biochemical reagents. Reagent-grade chemicals were obtained from either BDH, Fisher, or Sigma, and enzymes, as well as bacteriophage λ DNA (0.5 mg/ml), were obtained from Pharmacia. Hybond-N hybridization transfer membrane and [α-^{35}S]dATP were supplied by Amersham, and [α-^{32}P]dCTP (3,000 Ci/mmol [10 mCi/ml]) was obtained from ICN. Deoxyoligonucleotides were purchased from University Core DNA Services, The University of Calgary.

Field samples. All samples were obtained from oil fields in which oil was produced by water flooding. The oil-water mixture emerging at a production well in these fields is separated, and the produced water is reinjected into the field through injection wells. Water samples taken from production or injection wells will be referred to as production and injection waters, respectively. Production water and corrosion coupon samples, representing the planktonic and sessile microbial populations, were obtained from four saline production wells in the Wainwright field in eastern Alberta. The sampling protocols have been previously described (28, 30). A total of 24 samples were obtained and maintained under anaerobic conditions for the isolation of new bacteria.

Production and injection water samples used for monitoring the effect of nitrate on the microbial community were obtained from a comparable saline field, the Coleville oil field in southwestern Saskatchewan. Two injection water samples (1 liter each from I11-29 and I61-20) and two production water samples (2 liters each from 18-29 and 4-29) were taken 8 days prior to nitrate injection. These sites were then sampled 20, 55, and 82 days after nitrate injection was started. Samples were kept at ambient temperature and processed within 24 h of sampling.

Primary isolation culture media. Commercial medium for the culturing of acid-producing bacteria (APB) from saline waters was obtained from Bioindustrial Technologies, Inc. (Georgetown, Tex.). Media were prepared in the laboratory according to parallel fresh and saline recipes by inclusion of 2% (wt/vol) NaCl in the latter. Plate count agar (PCA) was prepared at full and quarter strength (PCA and 1/4 PCA, respectively) from Bacto PCA agar base (Difco). Eosin methylene blue (EMB) plates were prepared from Bacto EMB agar base (Difco) supplemented with lactose and sucrose (both at 5 g/liter). Blood agar (BA) plates were prepared from Bacto BA base (Difco) with sheep's blood. Butlin's B medium plus glucose (BG) was essentially prepared as described in reference 4 with the addition of 50 mM glucose.

Nitrate injection. Nitrate was injected in the Coleville field, located north of Kindersley, Saskatchewan, and owned and operated by Phillips Petroleum Company. The field was discovered in 1951, and the wells produce primarily out of the

Bakken sandstone formation at a depth of 900 m. The oil is a heavy, asphaltic crude with an API gravity near 13. The average permeability of the formation is 0.5 to 1.0 Darcies, with streaks of 2.0 to 3.0 Darcies. Water injection began in 1958 and continues today with over 90% of the water being reinjected produced water. The injected water is at a temperature of about 30°C and contains less than 8,000 mg of total suspended solids per liter, of which about 100 mg/liter is sulfides. The pH is 7.5 to 8.5 at atmospheric pressure.

Two water injection wells were used in this study, I11-29 and I61-20, along with 16 adjacent producing wells. The results from two of the producing wells, 18-29 and 4-29, separated from the injection wells by a distance of ca. 300 m, are reported in this study. The earliest water breakthrough time from injector to producer, as determined by a tritium tracer survey, was 1 to 2 weeks for both producers. A concentrate containing ammonium nitrate (Imperial Oil Products, Calgary, Alberta, Canada) and sodium phosphate (NaH_2PO_4; Van Waters and Rogers, Ltd., Saskatoon, Saskatchewan, Canada) was made up in freshwater at concentrations of 470 and 14.3 g/liter, respectively. This concentrate was pumped continuously into an injection manifold upstream of each injector on 25 June 1996. The distances from the manifold to the injector were 492 m for I61-20 and 100 m for I11-29. The residence times between the manifold and injection header were calculated to be 18 and 0.73 min, respectively. The in-line concentrations of ammonium nitrate and sodium phosphate were calculated as 400 and 12 mg/liter. From 25 June (day 0), injection continued for 50 days at an average rate of 38 liters/day for I61-20 and 190 liters/day for I11-29. The ammonium nitrate concentration was reduced by 33% at I61-20 on day 16, and this injector was shut down for 2 days during the 50-day injection period to repair a leaking tube.

Both injectors and producers were monitored for changes in sulfide, in sulfide-oxidizing, nitrate-reducing bacteria (NRB), and in SRB before, during, and after chemical injection. Samples were collected in 1.0-liter, sterile, screw-cap, glass, deoxygenated bottles sealed with a butyl-rubber septum. Sulfide was determined colorimetrically with a sulfide detection kit from Aquaquant (EM Sciences, Gibbstown, N.J.). SRB were detected by culture of brine in 10-ml serum bottles containing API RP-38 medium with 10 g of NaCl per liter (C&S Laboratories, Tulsa, Okla.), and NRB were detected by culture in serum bottles containing sterile-filtered (0.22-μm pore size) injection brine containing about 100 mg of sulfide per liter (pH 7.5) and amended with 5 mM NH_4NO_3, 0.1 mM NaH_2PO_4, and 0.01% resazurin (13, 14). Enumeration of SRB and NRB was performed by inoculation of a single series of eight bottles containing one of the media described above. Each series was inoculated with a 10-fold serial dilution of the sample. Bottles were incubated at room temperature for 5 days (NRB) or 3 weeks (SRB), with the highest dilution reported as the count in bacteria per milliliter. Positive NRB bottles were determined as a change in the appearance of the brine from colorless to pink due to an increase in redox potential (13).

Enrichment culture and colony purification of heterotrophs. All enrichments and plate incubations were at room temperature (22°C). Heterotrophic bacteria were purified from the Wainwright samples through enrichment culture and plating protocols.

Spore-forming bacteria were enriched by heating of each sample (30 ml at 80°C for 10 min) and inoculation of 7.5 ml of the heat-treated sample into 100 ml of anaerobic BG media. Cultures showing growth were plated on 1/4 PCA (incubated aerobically and anaerobically) and on BA plates (anaerobic incubation only).

APB were enriched by inoculation of 2.0 ml of each field sample into sealed, saline APB medium bottles and incubation of them for 12 to 72 h. At intervals, subsamples were diluted in saline phosphate buffer and plated onto PCA, 1/4 PCA, saline 1/4 PCA, (incubated both aerobically and anaerobically), EMB, and saline EMB (incubated aerobically only). Isolated colonies displaying different colony morphologies were selected, restreaked on the same medium, and incubated under the same conditions as those used for the original colony. This was repeated until a single, consistent morphology was obtained.

Liquid cultures derived from single colonies were grown in saline Trypticase soy broth (TSB; Difco), saline quarter-strength TSB, or saline EMB broth base (containing lactose and sucrose but lacking the dyes). These were used for microbiological characterization, DNA isolation, and storage in glycerol at −70°C.

DNA isolation from liquid cultures and production waters. DNA was extracted from 10-ml liquid cultures of colony-purified isolates as described previously (31) and dissolved in TE (10 mM Tris-HCl, 0.1 mM EDTA [pH 8]).

For isolation of total community DNA, production and injection water samples were centrifuged at 16,000 × *g* for 20 min at 4°C to collect solids, including bacteria. Pellets obtained from a single sample were resuspended in 10 to 15 ml of the supernatant, pooled, and recentrifuged. DNA was isolated from these pellets as described earlier (28), dissolved in TE, and stored frozen at −20°C.

All DNA preparations were then subjected to electrophoresis on 0.7% (wt/vol) high-gelling-temperature agarose to check concentration and quality and further purified by precipitation with an equal volume of 30% (wt/vol) polyethylene glycol (PEG 8000)–1.5 M NaCl (23). Following dissolution in TE, concentrations were estimated fluorimetrically (28).

Identification and characterization of bacterial standards. Following purification and concentration determination, 169 DNA preparations derived from purified strains were tested for genomic cross-hybridization by a dot blot procedure under high-stringency conditions (23, 31). Chromosomal DNA preparations with strong and identical reciprocal cross-hybridizations were pooled. A total of 40 different standards, defined as bacteria with genomes showing relatively little genomic cross-hybridization (28, 30, 31), were defined by cross-hybridization analysis of the original pool of 169 chromosomal DNAs. Sixteen of these, selected on the basis of DNA availability and reproducibility of regrown cultures, were further characterized with microbiological and molecular biological techniques and included in the RSGP analysis.

Colony and cell morphologies, as well as the Gram reaction with the potassium hydroxide test (24), were determined for all 16 heterotrophs. A selected number of heterotrophs were subjected to the following tests (8): acid-fast staining (Ziehl-Neelson method); endospore staining (Dorner method); and the oxidase, catalase, and/or nitrate reduction test. Eleven isolates were further identified with the API 20E manual biochemical system for microbial identification (Analytab Products) according to the manufacturer's instructions. The ability of aerobically cultured organisms to grow anaerobically and vice versa was determined. Nitrate reduction to nitrite or dinitrogen was tested independently of the API identification process (8).

A partial 16S rRNA gene sequence was determined for all newly isolated standards. A 1.4-kb fragment was amplified by PCR with primers f8 (17) and r1406 (11). Genomic DNA (4 μl; 20 ng/μl), 10× buffer (5 μl; 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl [pH 9]), $MgCl_2$ (2.5 μl; 50 mM), deoxynucleoside triphosphates (4.0 μl; 2.5 mM each), primers (1 μl each of f8 and r1406; 10 pmol/μl), and *Taq* polymerase (0.25 μl of 5 U/μl) were combined in a total volume of 50 μl. Amplification was in a Perkin-Elmer GeneAmp 2400 PCR system with two consecutive sets of 15 cycles. The first set of 15 cycles was 30 s at 94°C, 30 s at 60°C (with the temperature decreasing by 0.5°C per cycle), and 60 s at 72°C. This was immediately followed by 15 cycles of 30 s at 94°C, 30 s at 52°C, and 60 s at 72°C. After the two sets of cycles, 50 μl of TE and 60 μl of 20% (wt/vol) PEG 8000–2.5 M NaCl were added, and following incubation at 37°C for 10 min, the PCR product was collected by centrifugation. After being washed with 70% (vol/vol) ethanol and then drying, PCR products were dissolved in 10 μl of TE and visualized on 1% (wt/vol) high-gelling-temperature agarose gels. The PCR products were sequenced directly with the Promega fmol cycle sequencing system with EUB338 (2) and primer P76 (GCCAGC[A/C]GCCGCG GT) targeting conserved regions of the 16S rRNA (positions 338 to 356 and 517 to 531, respectively; *Escherichia coli* numbering). Identification in terms of the best-matching sequence was then done by searching the RDP database with the program SIMILARITY_RANK (15) or by searching the GenBank database with BLAST (1). Identifications based on microbiological characterization and the 16S rRNA sequences are summarized in Table 1.

Reverse genome probing. Chromosomal DNAs from 30 SRB (23, 28, 30, 31), the nitrate-utilizing bacterium CVO (26), and the 16 newly isolated heterotrophic standards were used. A survey of all 47 standards is given in Table 1. A batch of 200 master filters was prepared by spotting 2 μl of all denatured chromosomal DNAs on 8 by 7-cm Hybond-N grids at the concentrations indicated in Table 1. Low concentrations of Ace1,3, NH4, NH12, and NH31 were spotted because only limited amounts of chromosomal DNA were available at the time of master filter preparation. Denatured bacteriophage λ DNA (5, 10, 20, 30, 50, 100, 200, and 400 ng) was also included on the filters (28). Following covalent linkage of the DNAs to the filters (30), the filters were stored at −20°C.

Sample DNA (100 ng) and bacteriophage λ DNA (2.5 ng) were combined and labeled with [α-^{32}P]dCTP and the random hexamer procedure (28, 30). Labeled probes were hybridized with the master filters by a single high-stringency procedure (20, 29). After washing and drying, the dot blots were exposed to BAS-IIIS type Imaging plates which were scanned with a BAS1000 Bio Imaging Analyzer (Fuji). MacBas 2.2 software was used to determine hybridization intensities in units of photostimulable luminescence (PSL) for all of the dots. A local background level (the PSL of an adjacent area) was determined for all hybridization spots. PSL values were exported to Microsoft Excel 5.0 for further computational analysis. Net values (ΔPSL) were obtained by subtraction of the local background and were used for further calculations.

Quantitative analysis of hybridization data. Data were analyzed with equation 1 (28):

$$f_x = (k_\lambda/k_x) \times (I_x/c_x) \times (I_\lambda/c_\lambda)^{-1} \times (f_\lambda) \quad (1)$$

where k_x and k_λ are hybridization constants, f_x and f_λ are the weight fractions of standard x and bacteriophage λ DNA in the probe that hybridized with immobilized standards x and λ, c_x and c_λ are the weights of denatured DNAs x and λ spotted on the filter, and I_x and I_λ are the observed ΔPSL values. Linearity between I_λ and c_λ was generally observed up to $c_\lambda = 20$ ng, and the (I_λ/c_λ) values obtained for this range were averaged for all calculations. In initial experiments, chromosomal DNAs for individual standards were mixed with λ DNA, labeled, and hybridized with master filters to evaluate the ratio (k_λ/k_x):

$$(k_\lambda/k_x) = (f_x/f_\lambda) \times (I_\lambda/c_\lambda) \times (I_x/c_x)^{-1} \quad (2)$$

using $f_x = 0.976$ and $f_\lambda = 0.024$ (100 ng of DNA for standard x combined with 2.5 ng of λ DNA combined in the labeling reaction). Standards Ace1,3, NH4, NH12, and NH31 were evaluated with $f_x = 0.800$ and $f_\lambda = 0.200$ (10 ng of standard DNA combined with 2.5 ng of λ DNA). These studies also gave information on the degree of cross-hybridization between the chromosomal DNAs of all 47 standards used in this study.

TABLE 1. Identification and properties of standards present on the oil field master filter

Identification no.[a]	Standard	Oxygen use[b]	Identification[c]	Amt of DNA spotted on master filter (ng)	$<k_\lambda/k_x>$[d]	σ[e]
1	Lac1,2	Anaerobe	*Desulfovibrio vulgaris*	100	441	35
2	Lac3	Anaerobe	*Desulfovibrio desulfuricans*	100	303	80
3	Lac4	Anaerobe	*Desulfovibrio* sp.	100	667	247
4	Lac5	Anaerobe	*Desulfovibrio* sp.	100	1214	306
5	Lac6	Anaerobe	*Desulfovibrio* sp.	100	428	10
6	Lac7	Anaerobe	*Eubacterium* sp.	100	274	18
7	Lac10	Anaerobe	*Desulfovibrio* sp.	100	356	96
8	Lac12	Anaerobe	*Desulfomicrobium* sp.	100	219	29
9	Lac15	Anaerobe	*Desulfomicrobium* sp.	100	678	134
10	Lac21	Anaerobe	*Desulfovibrio* sp.	100	1,630	530
11	Lac22	Anaerobe	*Desulfovibrio* sp.	100	832	10
12	Lac23	Anaerobe	*Desulfomicrobium* sp.	100	212	20
13	Lac24	Anaerobe	*Desulfovibrio* sp.	120	79	8
14	Lac25	Anaerobe	*Desulfovibrio* sp.	100	347	79
15	Lac26	Anaerobe	*Desulfomicrobium* sp.	100	123	25
16	Lac29	Anaerobe	*Desulfovibrio* sp.	100	1,013	453
17	Lac30	Anaerobe	*Desulfovibrio* sp.	100	220	83
18	Sty1	Facultative	*Pseudomonas* sp. or *Vibrio* sp.	100	291	54
19	Eth3	Anaerobe	*Desulfovibrio* sp.	100	78	12
20	Ben1	Anaerobe	*Desulfovibrio* sp.	100	328	96
21	Ben3,4	Anaerobe	*Plesiomonas* sp.	100	336	29
22	Ben7	Anaerobe	*Desulfobacter* sp.	100	331	34
23	Dec4	Anaerobe	*Desulfovibrio* sp.	100	97	47
24	Dec6	Anaerobe	*Desulfoarculus* sp.	100	212	31
25	Pro4	Anaerobe	*Desulfobulbus* sp.	100	224	39
26	Pro5	Anaerobe	*Desulfobulbus* sp.	120	453	7
27	Pro12	Anaerobe	*Desulfococcus* sp.	100	381	74
28	Ace1,3	Anaerobe	*Desulfobacter* sp.	100	227	51
29	Ace3,4	Anaerobe	*Desulfobacter* sp.	10	128	4
30	Ace8	Anaerobe	Unidentified	100	337	3
31	Ace9	Anaerobe	Unidentified	100	2,314	620
32	CVO	Microaerophile	*Campylobacter* sp.	100	55	16
33	NH2	Facultative	*Vibrio* sp.	100	592	182
34	NH4	Aerobe	*Bacillus* sp.	10	640	117
35	NH6	Facultative	*Proteus* sp.	100	222	80
36	NH7	Facultative	*Aeromonas* sp. or *Shewanella* sp.	100	158	66
37	NH8	Facultative	*Bacillus* sp.	100	938	12
38	NH9	Anaerobe	*Bacillus* sp.	100	1,248	426
39	NH10	Anaerobe	*Bacillus* sp.	100	274	68
40	NH12	Facultative	*Streptococcus* sp.	10	115	26
41	NH15a	Facultative	*Citrobacter* sp. or *Salmonella* sp.	100	148	21
42	NH15b	Facultative	*Serratia* sp. or *Yersinia* sp.	100	858	236
43	NH19	Aerobe	*Nocardia* sp. or *Bacillus* sp.	100	437	111
44	NH23	Aerobe	*Vibrio* sp.	100	52	14
45	NH27	Aerobe	Marine eubacterium	100	121	53
46	NH30	Aerobe	*Oceanospirillum* sp.	100	256	44
47	NH31	Aerobe	Marine eubacterium	10	418	ND[f]

[a] Identification number corresponding to the position of the standard on the master filter.
[b] Ability to use oxygen and/or grow anaerobically. NH19 to NH31 were not tested for anaerobic growth.
[c] Genus derived from comparison of 16S rRNA sequence with the RDP database (15) and/or from data obtained with the API system.
[d] Average value for k_λ/k_x for duplicate measurements. The overall average was 453.
[e] Average deviation for duplicate measurements. The overall average was 103.
[f] ND, not determined. For NH31, only one measurement was done.

RESULTS

Isolation of oil field heterotrophs. Previous work with the microbial community present in western Canadian oil fields of moderate temperature and salinity, from which oil is produced by water injection, focused on SRB, particularly *Desulfovibrio* spp. (28, 30). However, analysis of 16S rRNA sequences indicated the additional presence of anaerobic fermentative and sulfide-oxidizing bacteria (26). One of these, CVO, which in consortia with other members of the injected brine derives energy from the reaction $5HS^- + 2NO_3^- + 7H^+ \rightarrow 5S^0 + N_2 + 6H_2O$ (14), was shown to be widely distributed in this environment (26). Although the presence of aerobic or facultative heterotrophs was not indicated by 16S rRNA sequencing, these heterotrophs were readily isolated when production water samples were plated, either directly or following enrichment, on PCA, BA, or EMB plates. The 16 isolates, 6 gram positive (NH4, NH8, NH9, NH10, NH12, and NH19) and 10 gram negative, that were added to the master filter for RSGP analysis are listed in Table 1. Three (NH15b, NH23, and NH31) were found to be able to produce nitrite from nitrate. Ten (Sty1, NH2, NH4, NH6, NH8, NH12, NH15a, NH15b, NH19, and NH23) were tentatively identified at the genus level with

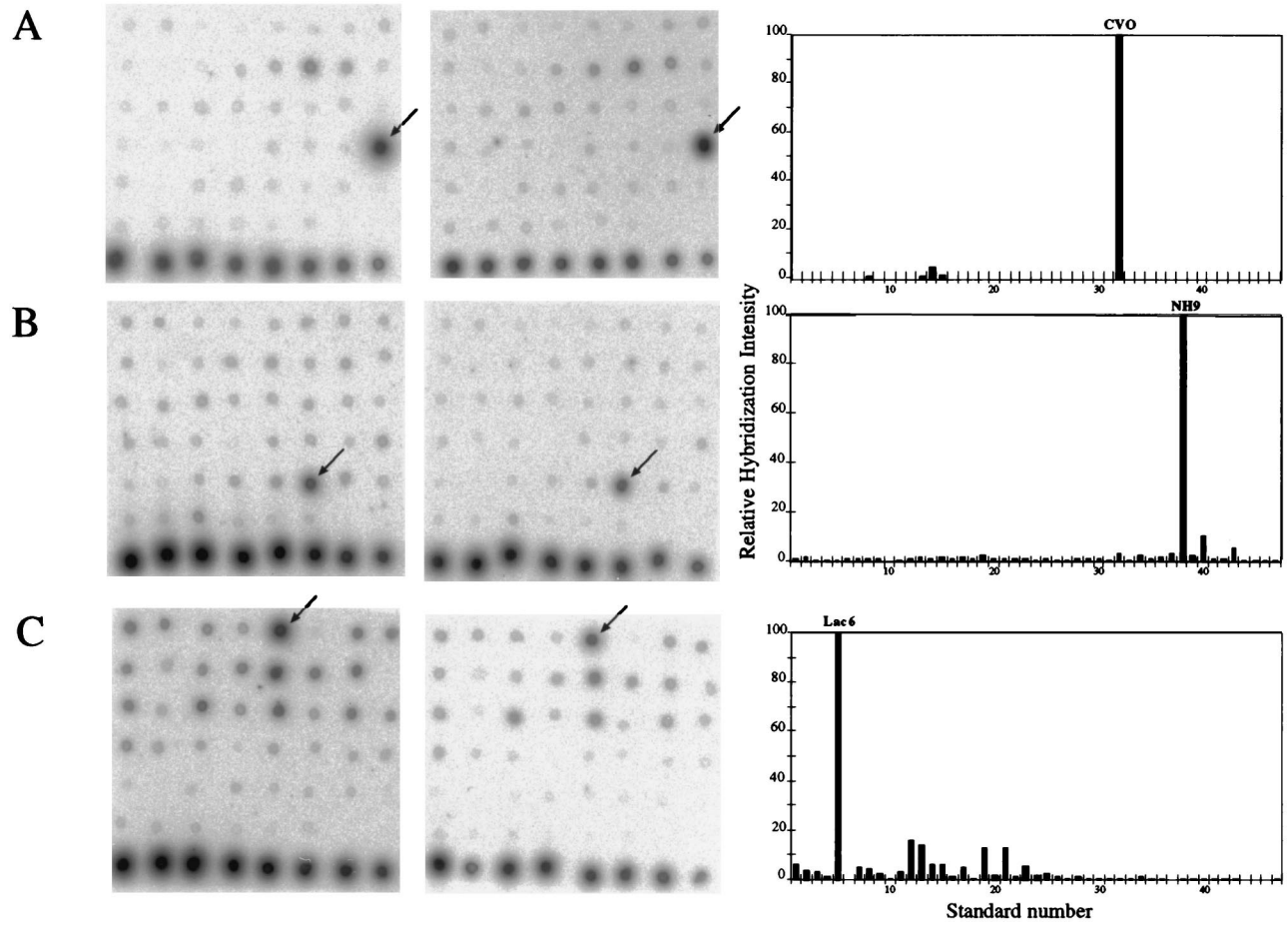

FIG. 1. Hybridization of standard DNAs with the oil field master filter. The chromosomal DNAs for standards 1 to 47 were present in the first six rows in the order indicated in Table 1. The bottom row contained (from left to right) decreasing amounts of bacteriophage λ DNA. A mixture of 2.5 ng of bacteriophage λ DNA and 100 ng of chromosomal DNA was labeled and hybridized with the master filter in duplicate as shown. The results of duplicate hybridizations were averaged and are displayed in a bar diagram. (Relative I_x is on the *y* axis; self-hybridization is taken as 100%, and the standard number is given on the *x* axis.) Data are shown for standard 32 (CVO) (A), standard 38 (NH9) (B), and standard 5 (Lac6) (C). The arrow indicates self-hybridization.

the API system and additional tests as *Pseudomonas* sp., *Vibrio* sp., *Bacillus* sp., *Proteus* sp., *Bacillus* sp., *Streptococcus* sp., *Citrobacter* sp., *Serratia* sp., *Nocardia* sp., and *Vibrio* sp., respectively. These identifications agreed with those derived by partial 16S rRNA sequencing in the case of NH2, NH4, NH6, NH12, and NH23. The two identification methods (API and 16S rRNA) indicated different genera within the family *Enterobacteriaceae* for NH15a (*Citrobacter* sp. or *Salmonella* sp.) and NH15b (*Serratia* sp. or *Yersinia* sp.). The 16S rRNA identifications of these 16 isolates (Sty1 and NH2 to NH31) are listed in Table 1, together with those for 29 of 31 previously described standards (SRB and CVO).

Hybridization of standard DNAs. The application of phosphorimaging plate technology allows rapid, sensitive, and reliable quantitative analysis of hybridization patterns obtained by RSGP. In a previous study, the fractions f_x of the standard genomes present in an environmental sample were obtained from film densitometric data by equation 1 and $(k_\lambda/k_x) = 102$, the average value found for standards Lac3 and Lac6 (28). In this study, we determined (k_λ/k_x) values for all 47 standards in duplicate by labeling a defined mixture of standard and λ DNA and hybridizing the resulting probe with the master filter. Some representative results are shown in Fig. 1. The averages of the duplicate (k_λ/k_x) values are presented in Table 1 and range from 55 for CVO to 2,314 for Ace9. These values reflect different apparent genome complexities; i.e., when 100 ng of CVO DNA ($k_\lambda/k_x = 55$) is labeled together with 0.25 ng of λ DNA (Fig. 1A), the hybridization intensity I_x is similar to I_λ for the largest amount of λ DNA spotted ($c_\lambda = 400$ ng), whereas when a mixture of NH9 ($k_\lambda/k_x = 1{,}248$) and λ DNA is labeled (Fig. 1B), I_x corresponds to the low end of the I_λ scale ($c_\lambda = 5$ ng). Complete quantitative analysis of the hybridization patterns also allowed the degree of cross-hybridization between the test genome and the 46 other genomes on the filter to be determined. Data for the chosen examples are shown in Fig. 1 as a bar diagram averaged for the two hybridization patterns. Cross-hybridizations were generally low relative to self-hybridization, which was taken as 100%. CVO only showed significant cross-hybridization with Lac25 (5%), whereas all other cross-hybridization levels were below 2%. The Lac6 genome showed significant cross-hybridization with genomes of other *Desulfovibrio* spp. (Fig. 1C [up to 14%]) in a reproducible pattern. All cross-hybridizations are displayed in a matrix for all 47 standard genomes in Fig. 2, where each square is shaded in proportion to the percentage of cross-hybridization. Strong cross-hybridization was also observed between the genomes of Lac15 and Lac26 and Lac29 and Lac15 (Fig. 2). An anomalous hybridization pattern was found for standard NH4, which hy-

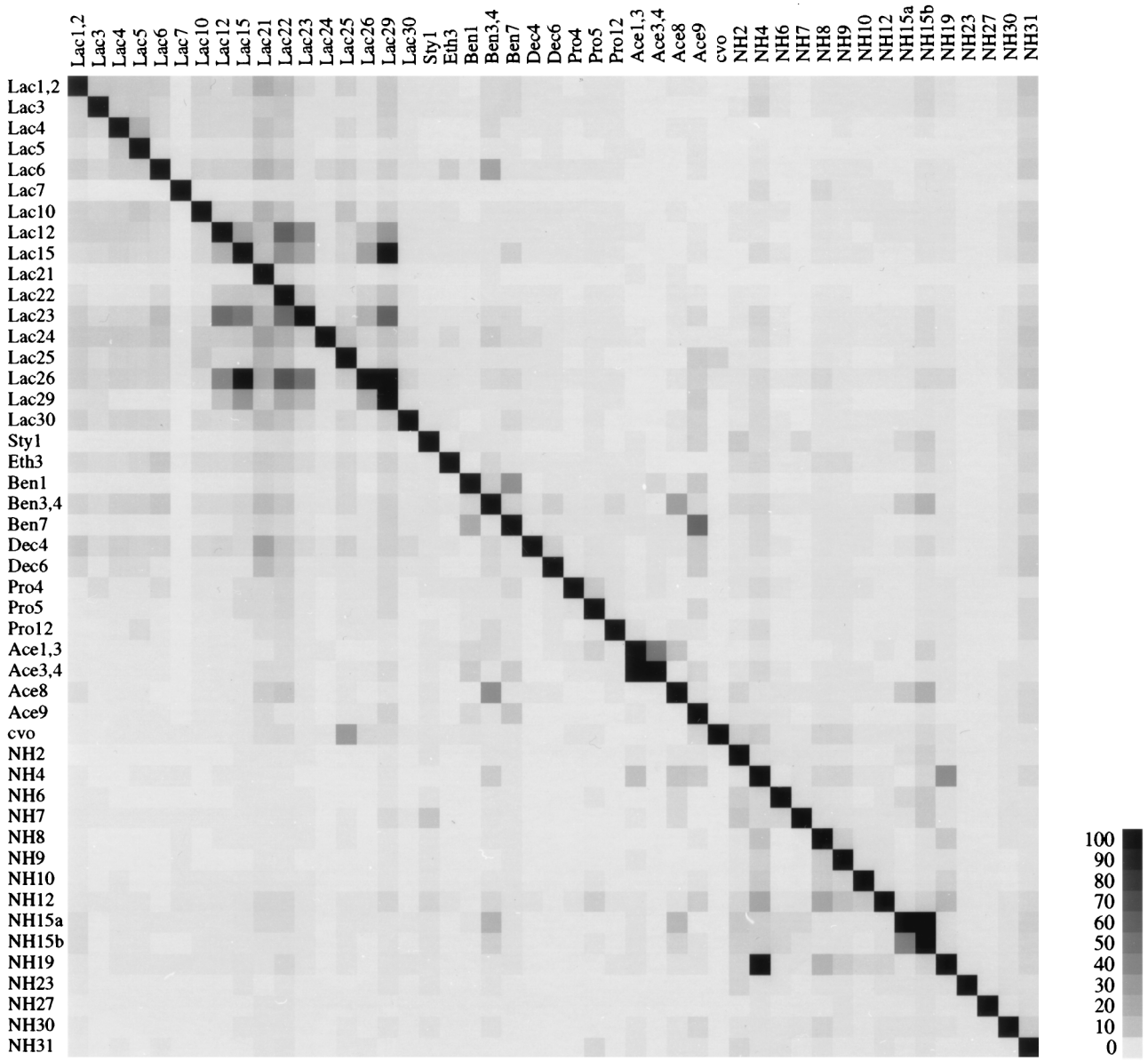

FIG. 2. Cross-hybridization matrix for the genomes of all 47 standards. The columns represent the results of duplicate hybridization experiments. Each square in the matrix is shaded in proportion to its percentage of cross-hybridization with the DNA on the diagonal square in the column, according to the gray scale provided. Self-hybridizations (the squares on the diagonal) were taken as 100%.

bridized stronger with NH19 than with itself (Fig. 2). The data in this matrix can in principle be used to correct hybridization data for cross-hybridization.

As a test of the validity of equation 1 for calculating fractions of standard genomes with the (k_λ/k_x) values listed in Table 1, defined mixtures of standard genomes were analyzed. Representative examples of results obtained for mixtures of equal weights of two, three, or five standard genomes are shown in Fig. 3. The actual composition of the mixture is shown as a bar diagram (f_x versus standard number), together with the bar diagram expected in view of the known cross-hybridization matrix. The latter patterns can be compared with those actually observed, and these can in turn be corrected for cross-hybridization (Fig. 3) by subtraction of the contributions to I_x resulting from cross-hybridization with main peaks (e.g., as in Fig. 1). The results for all studies are summarized in Table 2. Actual and experimentally determined f_x values generally agreed well. Systematic differences could be caused either by differential labeling of λ and the chromosomal sample DNA or by pipetting errors when these two DNAs are combined. For instance for mixture 11 (Table 2), the actual f_x values (0.86, 0.1, and 0.02) differed by about a factor of 2 from those calculated from the hybridization pattern, either before (0.49, 0.07, and 0.01) or after (0.48, 0.05, and 0.01) correction for cross-hybridization. More reliable absolute values of f_x were obtained if these were calculated from the averages of duplicate hybridizations. However, even for single hybridizations of DNAs from mixtures or environmental samples, the patterns obtained were highly reproducible, except for the absolute values on the *y* axis.

Nitrate injection in an oil field: chemical and microbiological data. Within 20 days of nitrate injection, the sulfide concentrations declined substantially at both injector and produc-

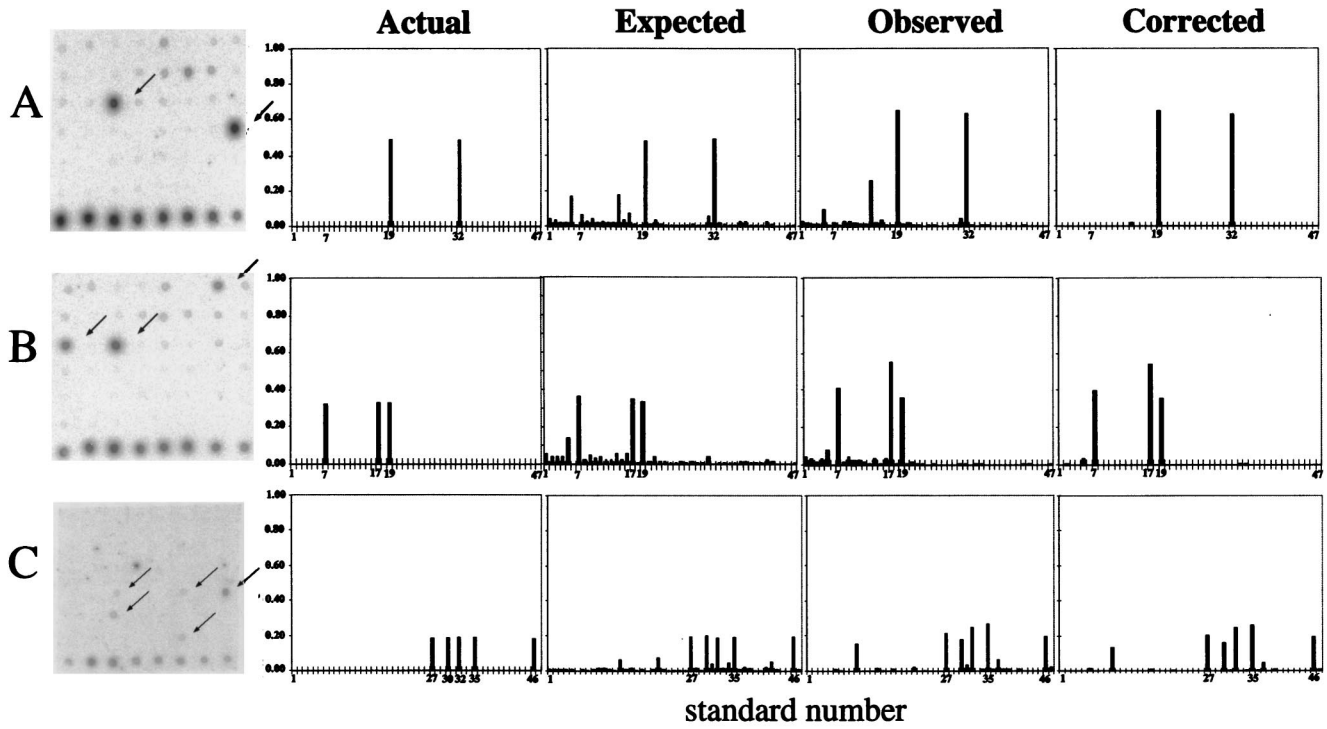

FIG. 3. Analysis of mixtures of standard genomes. Standard genomes were mixed in the indicated ratios (Actual). The expected bar diagram (f_x versus standard number) calculated from the cross-hybridization matrix (Fig. 2) is shown (Expected). Hybridization of the mixed genome probe with the master filter gave the patterns shown in panels A, B, and C; the arrows indicate the genomes that were mixed together. The bar diagram derived from this hybridization pattern by equation 1 is shown both before and after correction for cross-hybridization (Observed and Corrected, respectively).

ing wells (Table 3). The counts of NRB increased dramatically, while those of SRB remained relatively unchanged during this period. At day 55, 5 days following shutdown of nitrate injection, sulfide concentrations increased sharply at I61-20, while NRB counts declined at both injectors and producers. Sulfide concentrations at both producers declined to their lowest point (8 and 16 mg/liter), less than 25% of the levels measured prior to nitrate injection. Low sulfide levels are still expected at the producing wells 5 days following cessation of nitrate injection in view of the breakthrough times of 7 to 14 days between injectors and producers. At day 82, sulfide concentrations and NRB counts were near the levels before treatment at both injectors, while SRB were slightly below the counts before treatment. Both producers also showed a slight drop in SRB counts and an increase in sulfide levels at this time. NRB counts decreased slightly from levels measured on day 55, but remained well above values prior to nitrate injection at both production wells. These results suggest that the nitrate injection had some residual activity in controlling SRB activity at both injectors and producers.

Effect of nitrate on the microbial community in an oil field. The effects of nitrate injection on the microbial community in the oil field were monitored with RSGP. DNA was isolated directly from production or injection waters obtained 8 days before nitrate injection and 20, 55, and 82 days after nitrate injection (Fig. 4, −8, 20, 55, and 82, respectively). Hybridiza-

TABLE 2. Model mixtures used for hybridization with the master filter

Identification no.	Components	f_x of components		
		Actual[a]	Observed[b]	Corrected[c]
1	Lac1,2; Lac3	0.33; 0.66	0.48; 0.36	0.43; 0.34
2	Eth3; CVO	0.49; 0.49	0.65; 0.63	0.50; 0.54
3	Dec4; Dec6	0.49; 0.49	0.67; 0.87	0.47; 0.58
4	Lac10; Lac30	0.49; 0.49	0.42; 0.54	0.36; 0.36
5	Lac10; Lac30; Eth3	0.33; 0.33; 0.33	0.41; 0.55; 0.37	0.36; 0.53; 0.36
6	Sty1; Lac7	0.66; 0.33	0.43; 0.23	0.42; 0.22
7	NH30; NH6; Ace8; Pro12; CVO	0.19; 0.19; 0.19; 0.19; 0.19	0.20; 0.27; 0.18; 0.21; 0.25	0.19; 0.20; 0.20; 0.19; 0.20
8	Lac22; Lac26; Ace8; NH4	0.3; 0.4; 0.25; 0.02	0.34; 0.33; 0.22; 0.02	0.32; 0.19; 0.21; 0
9	Ace8; NH15a; NH12	0.1; 0.68; 0.2	0.08; 0.88; 0.17	0; 0.15; 0.42
10	Lac5; Lac10; Pro5; NH19	0.1; 0.49; 0.1; 0.3	0; 0.41; 0.06; 0.29	0; 0.41; 0.06; 0.29
11	Lac22; Ben3,4; Ace1,3	0.86; 0.1; 0.02	0.49; 0.07; 0.01	0.48; 0.05; 0.01

[a] Ratio of genomic DNAs mixed together; the balance is λ DNA.
[b] Values for f_x calculated from the hybridization pattern with equation 1; the data are calculated for a single hybridization.
[c] Values for f_x corrected for cross-hybridization.

TABLE 3. Sulfide concentrations and SRB and NRB counts measured at injectors and producers before and after injection of nitrate

Time (days)[b]	Concn in[a]:											
	Injector[c]						Producer[d]					
	I11-29			I61-20			18-29			4-29		
	Sulfide (mg/liter)	SRB (ml^{-1})	NRB (ml^{-1})	Sulfide (mg/liter)	SRB (ml^{-1})	NRB (ml^{-1})	Sulfide (mg/liter)	SRB (ml^{-1})	NRB (ml^{-1})	Sulfide (mg/liter)	SRB (ml^{-1})	NRB (ml^{-1})
−8	104	10^4	10^5	112	10^4	10^5	35	10^3	0	68	10^2	0
19	64	ND[e]	10^8	0	ND	10^8	16	10^2	10^6	48	10^3	10^7
20	64	ND	ND	0	ND	ND	14	ND	ND	40	ND	ND
26	64	10^4	10^8	1.2	10^4	ND	14	10^2	10^7	40	10^2	10^7
55	64	10^2	10^5	64	10^3	10^6	8	10^2	10^4	16	10^2	10^4
82	104	10^2	10^3	104	10^2	10^4	20	10^1	10^3	64	10^1	10^3

[a] Nitrate injection was for 50 days, from day 0 to day 50, as explained in the text. The breakthrough time from injector to producer wells was 7 to 14 days.
[b] Days following the start of nitrate injection at day 0.
[c] Sulfide readings at day −8 were measured 8 days prior to nitrate injection.
[d] Sulfide readings reported for day −8 are averages of three to four readings made prior to the estimated breakthrough time for nitrate.
[e] ND, not determined.

tion of labeled community DNAs with the master filters and data analysis gave the bar graphs shown in Fig. 4. It is clear from the observed patterns that standard 32 (CVO) was the dominant community member in samples collected 20 days after nitrate injection with a calculated f_x of 0.3 to 1.2 both in injection wells (Fig. 4, I11-29 and I61-20) and in production wells (Fig. 4, 4-29 and 18-29). None of the SRB represented on the filter showed a significant increase, with the exception of

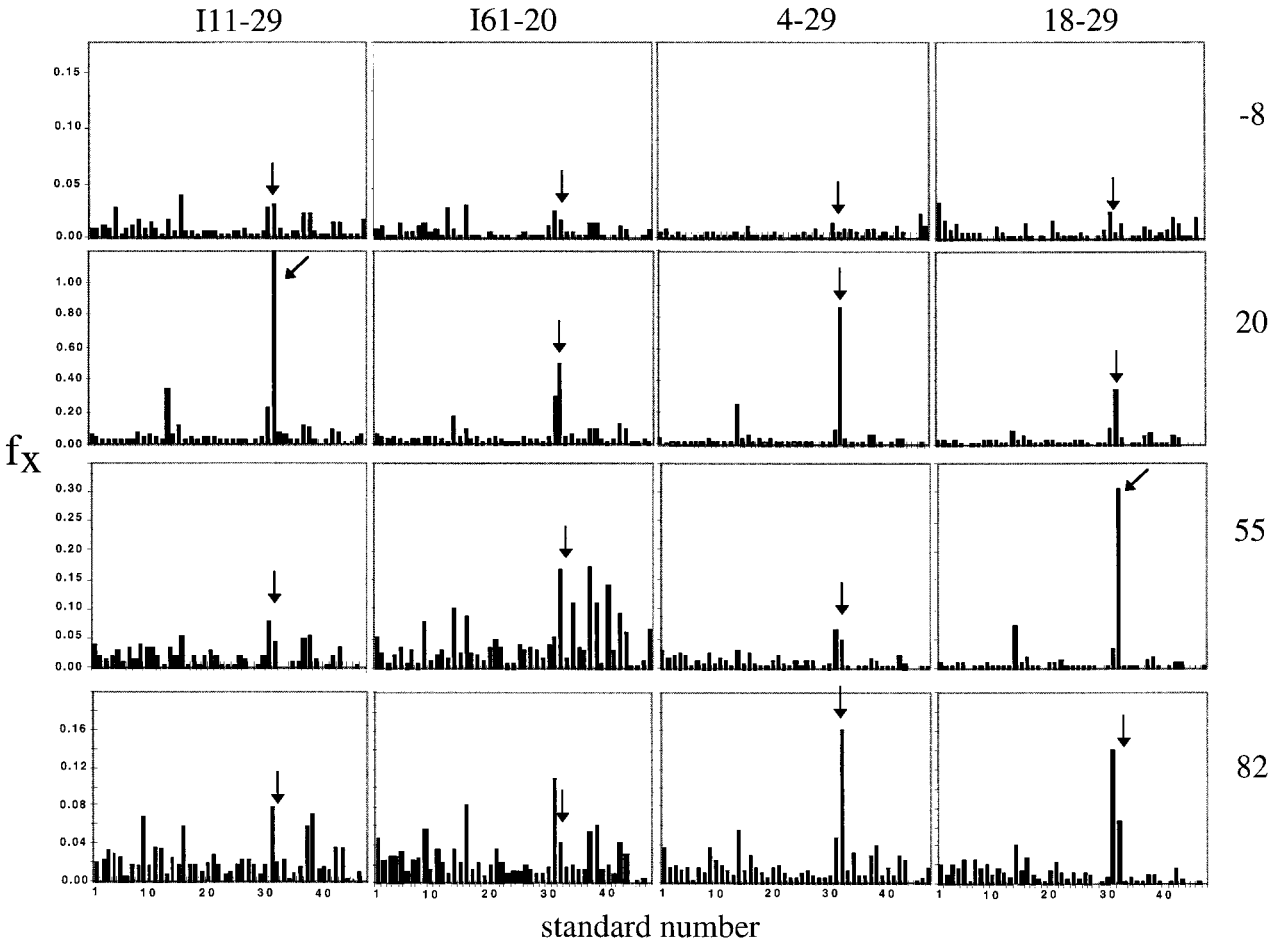

FIG. 4. Influence of nitrate injection on the microbial community in an oil field. Total DNA was isolated from water samples obtained from two injection wells (I11-29 and I61-20) and two production wells (4-29 and 18-29). The DNAs were labeled and hybridized with the master filter. The hybridizations observed are displayed as bar diagrams (f_x on the vertical axis versus standard number on the horizontal axis; data not corrected for cross-hybridization) for samples obtained before (−8 days) or after (20, 55, or 82 days) nitrate injection was initiated, as indicated on the left. Nitrate injection was stopped at day 50. The arrows indicate hybridization of CVO.

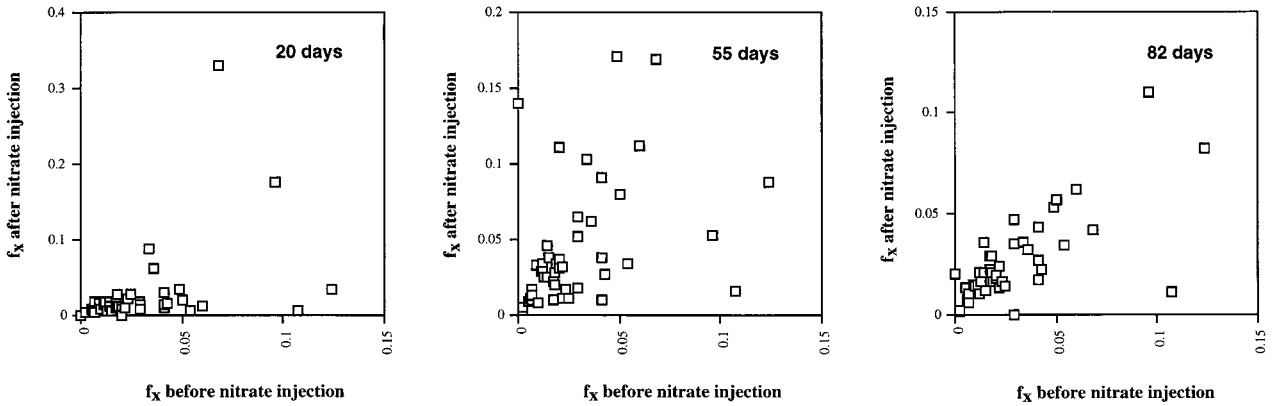

FIG. 5. Relaxation of the microbial community to that present before nitrate injection. The fractions f_x for community members before injection (Fig. 4, −8) are plotted against those after injection (Fig. 4, 20, 55, and 82) for injection well I61-20. The data approach a straight line with slope $m = 1$ as time progresses.

standard 14 (Lac25). However, this increase can be attributed to cross-hybridization (Fig. 1A). Also, none of the heterotrophs isolated in this study, of which three were shown to produce nitrite from nitrate (standards 42, 44, and 47), showed a strong increase. It thus appears that of the community members represented on the filter, CVO is the primary benefactor from nitrate addition. The fraction f_x calculated for CVO decayed to the levels observed prior to nitrate injection in the injection wells, but remained higher in the production wells (Fig. 4, 55 and 82 days, and Fig. 5).

DISCUSSION

As demonstrated in this paper, RSGP can be used to monitor the response of microbial communities to environmental changes, such as the sudden availability of the high-potential electron acceptor nitrate. The method was used previously to demonstrate that the SRB population on oil field corrosion coupons differed from that in the planktonic phase by bias towards certain *Desulfovibrio* spp., which were thought to be important in catalyzing anaerobic microbial corrosion (28). The use of phosphorimaging plate technology greatly accelerated the quantitation of hybridization signals, which is now routinely done for all hybridization experiments. This allowed evaluation of all relative hybridization constants (k_λ/k_x) and all cross-hybridization coefficients (Table 1 and Fig. 2). The reasons for the wide variation in (k_λ/k_x) values are currently unknown. It is unlikely that this is caused only by differences in genome size. Values at the high end of the scale may result if a standard is not a pure culture, while values at the low end may reflect the presence of repetitive sequences (e.g., a small plasmid in high copy number). A typical bacterium has a value (k_λ/k_x) = 453 (the average of all data in Table 1). The very high value for standard 31 (Ace9) may be an artifact and may cause overestimation of its f_x (Fig. 4).

Although correction for cross-hybridization is meaningful for a closed system that consists of a mixture of known strains (e.g., a synthetic microcosm), its use for analysis of samples obtained from the environment ("open systems") is doubtful, because not all component chromosomes of the "environmental genome" can be obtained in pure form, e.g., by culturing. Thus the cross-hybridization matrix that should be used to correct data obtained for environmental samples remains incompletely known.

The data in Fig. 4, which for this reason were not corrected for cross-hybridization, indicate, when considered together with those in Table 3, that CVO can derive energy for growth from the oxidation of sulfide by nitrate in an oil field in situ. Sequencing of its 16S rRNA has indicated that CVO is most closely related to free-living *Campylobacter* spp. (26). *Sulfurospirillum deleyianum*, for which the physiology and taxonomy have been studied in detail (6, 21), is a similar organism. *S. deleyianum* has been shown to oxidize sulfide to sulfur with nitrate as an electron acceptor in two phases: (i) quantitative conversion of nitrate to nitrite followed by (ii) further reduction of nitrite to ammonia (6). Although the organism can derive energy for growth from a large number of redox reactions, Eisenmann and coworkers (6) believe that this sulfide-coupled nitrate ammonification may "represent an ecologically significant process in dark anoxic habitats with low redox potential." Oil fields certainly belong in the latter category, and we have shown that large-scale injection of nitrate in such a habitat greatly increases the presence of a similar organism. CVO is not identical to *S. deleyianum*, because its product of sulfide-dependent nitrate reduction is likely to be nitrogen, not ammonia (14). Eisenmann et al. (6) also suggested that other bacteria capable of using nitrate (e.g., denitrifying bacteria) may be less competive than *S. deleyianum* because of a reduced affinity for nitrate.

The primary goal of nitrate addition to an oil field is to decrease the amount of sulfide present. The feasibility of this goal had been demonstrated earlier in laboratory studies (9, 14) and has now been shown to be reachable in the field. The field data indicated that soluble sulfide measured at injection well I61-20 decreased from 112 mg/liter to undetectable levels 20 days after nitrate injection. Bottle counts of sulfide-oxidizing NRB indicated an increase from 10^5 to 10^8 bacteria per ml of injection water during this period with more dramatic increases measured at the production wells (Table 3). These data parallel the large increase seen in the fraction of CVO in the microbial community by RSGP after 20 days (Fig. 4). The RSGP results indicate that none of the SRB or heterotrophic bacteria that are represented on the master filter become dominant community components following injection of nitrate. These apparently do not use nitrate under in situ conditions, possibly because they cannot effectively compete for this electron acceptor, which is not normally present. The response of the oil field microbial community to nitrate injection seen in Fig. 4 and the field data in Table 3 justifies the conclusion that the primary use of the added nitrate is CVO-mediated sulfide oxidation and not hydrocarbon oxidation, as suggested in a model presented previously (26). In this model, sulfate or

other oxidized forms of sulfur were suggested as the most likely primary electron acceptors for hydrocarbon oxidation in the oil fields studied here (26). Anaerobic, sulfate-mediated hydrocarbon degradation has been directly demonstrated in other environments (5, 19). Following decay of the nitrate spike, the microbial community at the injection wells relaxes to its earlier composition. This can be demonstrated by plotting the f_x values obtained prior to injection against those following injection. The resulting plots are initially highly skewed but revert to a straight-line relationship with a slope of 1 as shown for one of the injection wells in Fig. 5. Thus, short-term injection of nitrate into an oil field can reduce sulfide levels without a concomitant increase in SRB activity. If nitrate were to be continuously added to injection waters, the emergence of a different microbial community with members capable of deriving energy directly from nitrate-dependent hydrocarbon oxidation may be anticipated.

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