APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1993, p. 4101–4114
0099-2240/93/124101-14$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 59, No. 12

Quantitative Reverse Sample Genome Probing of Microbial Communities and Its Application to Oil Field Production Waters

GERRIT VOORDOUW,[1,2]* YIN SHEN,[1] CLARE S. HARRINGTON,[1]† ANITA J. TELANG,[1] THOMAS R. JACK,[2] AND DONALD W. S. WESTLAKE[3]

Division of Biochemistry, Department of Biological Sciences, The University of Calgary, Calgary, Alberta, Canada T2N 1N4[1]; Novacor Research and Technology Corporation, Calgary, Alberta, Canada T2E 7K7[2]; and Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9[3]

Received 6 July 1993/Accepted 20 September 1993

This paper presents a protocol for quantitative analysis of microbial communities by reverse sample genome probing is presented in which (i) whole community DNA is isolated and labeled in the presence of a known amount of an added internal standard and (ii) the resulting spiked reverse genome probe is hybridized with a master filter on which denatured genomic DNAs from bacterial standards isolated from the target environment were spotted in large amounts (up to 1,500 ng) in order to improve detection sensitivity. This protocol allowed reproducible fingerprinting of the microbial community in oil field production waters at 19 sites from which water and biofilm samples were collected. It appeared that selected sulfate-reducing bacteria were significantly enhanced in biofilms covering the metal surfaces in contact with the production waters.

Reverse sample genome probing (RSGP) allows identification of bacteria in environmental samples by genomic DNA hybridization. In RSGP analysis, denatured chromosomal DNAs from bacteria obtained from the target environment (e.g., an oil field) are spotted on a master filter. DNA prepared from an environmental sample is then labeled and hybridized with the filter to identify which of the bacterial genomes spotted on the master filter are most prevalent in the sample. Ideally, the bacterial genomes spotted on the filter should show little or no cross-hybridization with each other, and isolates which meet this criterion are referred to as bacterial standards. These standards can be obtained from culture collections or by applying enrichment and colony purification procedures to samples obtained from the environment under investigation.

The method, as presented in earlier work (20, 21), was used to analyze the diversity of sulfate-reducing bacteria (SRB) in samples obtained from oil fields after liquid culture enrichment. The data obtained with this procedure (RSGP with growth) indicated that either of two distinct SRB communities, the saline and freshwater communities, was present in oil fields in western Canada. Although the RSGP with growth method is useful for qualitatively identifying bacterial standards present in liquid enrichment cultures, the results cannot be interpreted quantitatively, since growth in the laboratory shifts the community composition away from that present in the field (e.g., the media used in the earlier study were formulated by Pfennig et al. [12] to selectively enhance SRB).

Quantitative analysis of the microbial community requires that DNA, directly prepared from an environmental sample, be labeled and hybridized with the master filter. In this article, we demonstrate the feasibility of the RSGP without growth method and present its theoretical basis. The RSGP without growth method is then used for rapid quantitative fingerprinting of the microbial communities in two oil fields in central Alberta, Canada.

MATERIALS AND METHODS

Biochemical reagents. The Klenow fragment of DNA polymerase I, used for primer extension labeling, was obtained from Pharmacia. Hybond-N hybridization transfer membrane was supplied by Amersham, and [α-^{32}P]dCTP (3,000 Ci/mmol [10 mCi/ml]) was supplied by ICN. Random hexadeoxyoligonucleotides were supplied by the DNA synthesis laboratory of the University of Calgary. Reagent-grade chemicals were obtained from either Fisher or Sigma. Bacteriophage λ DNA (0.5 mg/ml) was obtained from Pharmacia and was used without further purification.

Field samples. Production water and biofilm samples were obtained from 19 sites in two fields, Wainwright (WW) and Wildmere (WM), in eastern Alberta (see Fig. 1 of reference 20). These reservoirs are shallow (600 m deep) and have a resident temperature of approximately 25°C. Oil is produced from these reservoirs by water flooding. A mixture of oil and water, containing 6% (wt/vol) dissolved salts, is separated in different production units, each consisting of a free water knockout (FWKO) in which the mixture is separated at an elevated temperature (20 to 50°C) and a water plant (WP) at approximately 20°C, in which the produced water is collected prior to reinjection into the reservoir. Water production rates varied from 200 to 1,200 m^3/day in the different units, and the residence time of produced water in the FWKO and WP varied from 3 to 24 h. Samples were obtained from eight production units in the WW field (WW1, WW5, WW6, WW13, WW14, WW20, WW28, and WWTP [WW truck pit]), each consisting of an FWKO and a WP. An unusual production unit was the WWTP, to which, in addition to oil-water mixtures from the reservoir, oil-water mixtures from distant locations were trucked for processing. Truck pit production waters thus may not be representative of the WW reservoir. Samples were obtained from a single

* Corresponding author.
† Present address: Department of Molecular and Cell Biology, Marischal College, University of Aberdeen, Aberdeen AB9 1AS, Scotland.

production unit in the WM reservoir, which, in addition to an FWKO (WM FWKO), consisted of an upper and a lower water plant (WM UWP and WM LWP, respectively).

Water samples (1 liter each) from 17 sites (WW1 FWKO, WW1 WP, WW5 FWKO, WW5 WP, WW6 FWKO, WW6 WP, WW14 FWKO, WW14 WP, WW20 FWKO, WW20 WP, WW28 FWKO, WW28 WP, WWTP FWKO, WWTP WP, WM FWKO, WM UWP, and WM LWP) were collected in plastic bottles, closed with a screw cap, and transported to the laboratory at ambient temperature. Samples from all 17 sites were collected four times at 2-week intervals (referred to as weeks 1, 3, 5, and 7 in Results) during May to July 1992. Sample processing was started within 24 h of sample collection.

Biofilm samples were obtained from the same sites, as well as from WW13 WP and WW13 FWKO, in five batches during the period from September to December 1992. For biofilm sampling, metal plugs with a 3.5-cm^2 surface area were removed from the sites. These plugs had been allowed to acquire a biofilm for 4 weeks. The plugs were transported to the laboratory at ambient temperature in 100-ml plastic bottles, together with 30 to 50 ml of production water from the sampling site. Although samples were obtained from all sites, a single sample batch contained samples for maximally 10 of the 19 sampling sites. Because multiple samples were obtained only from a limited number of sites (WW5 WP, WW6 FWKO, WW6 WP, and WW14 WP), time-dependent changes could not be analyzed. Dates will therefore not be given in the presentation of results for biofilm samples.

DNA isolation from planktonic samples. Solids including bacteria were collected from production water samples by centrifugation (23°C, 10 min, 16,000 × *g*). The final pellet was suspended in 1 ml of 0.15 M NaCl–0.1 M EDTA (pH 8) and stored frozen. Suspension in NaCl-EDTA and freezing apparently led to appreciable cell lysis; when 10-μl aliquots were electrophoresed on a 0.7% (wt/vol) high-gelling-temperature (HGT) agarose gel, 80 to 100% of the DNA migrated into the gel, with the balance remaining in the wells. Total planktonic DNA concentrations (see Results) were estimated by comparing the fluorescence intensity of the high-molecular-weight chromosomal DNA band of the samples with those of known amounts of *Hin*dIII-digested bacteriophage λ markers (21). Approximately 500 ng of DNA was then purified from aliquots of the frozen stock solutions with the Marmur procedure (9), modified to include digestion with both RNase A and proteinase K (18). Purified DNAs were dissolved in Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA [pH 8]). For determination of the concentration of DNA, 2-μl volumes of these purified DNA solutions, as well as 2-μl volumes of bacteriophage λ DNA solutions (2.5, 5, 10, 20, and 40 ng/μl), were spotted onto 20 ml of 1% (wt/vol) HGT agarose solidified in a petri dish and containing 3 μl of 0.5% (wt/vol) ethidium bromide. After 2 h at 22°C, the petri dish was placed on a UV transilluminator (8,000 μW/cm^2, 312 nm) and photographed. Concentrations were determined by visual comparison of fluorescence intensities with those observed for the λ standards. Reasonable accuracy (±10%) was obtained by determining the concentrations for two different dilutions of the purified DNA solution.

DNA isolation from biofilm samples. The biofilm from a 3.5-cm^2 removable metal plug was scraped into 7.5 ml of mineral salts solution (0.5 g of KH_2PO_4, 1.0 g of NH_4Cl, 4.5 g of Na_2SO_4, 0.06 g of $CaCl_2 \cdot 6H_2O$, 0.06 g of $MgSO_4 \cdot 7H_2O$, 3.5 ml of 0.1% [wt/vol] resazurin, 0.2 g of ascorbate, and 10.0 g of NaCl). Usually, biofilm scrapings from at least two plugs were combined for DNA purification by the modified Marmur procedure (18). Purified DNA preparations were dissolved in 25 μl of Tris-EDTA per plug. The quality of these DNAs was not suitable for primer extension labeling. Further purification was achieved by preparative electrophoresis through 0.7% (wt/vol) HGT agarose in 0.04 M Tris acetate–0.002 M EDTA (pH 8). Chromosomal DNA was then isolated from the gel after melting of the agarose in a boiling water bath, phenol extraction to remove the agarose, and ethanol precipitation. The resulting single-stranded DNA preparations were dissolved in 15 μl of Tris-EDTA per plug. The concentrations of these preparations were determined, as described for DNA preparations from planktonic samples, by using serial dilutions of either heat-denatured single-stranded λ DNAs or serial dilutions of native double-stranded λ DNAs as the reference. In the latter case, the estimated concentrations had to be multiplied by an experimentally determined correction factor of 1.5.

Preparation of reverse genome probes. DNA preparations were diluted to 10 ng/μl with Tris-EDTA and labeled with a primer extension protocol (20) in which 10 μl of a diluted DNA preparation and 10 μl of bacteriophage λ DNA (10 pg/μl) were combined with 6 μl of primer extension mix (20), 2 μl of Klenow polymerase (2 U/μl), and 2 μl of [α-^{32}P]dCTP. The λ DNA served as the internal hybridization standard, as explained below. After reaction for 3 to 5 h at 22°C, the resulting spiked reverse genome probe was boiled and hybridized with a prehybridized master filter for 16 h with the high-stringency procedure (18, 21). Up to 15 probes were prepared simultaneously. When DNA preparations with a concentration of <10 ng/μl were labeled, corrections were made for the changed ratio of the sample DNA to the internal standard.

Master filter preparation. Denatured chromosomal DNAs for 20 different standards, 16 SRB and 4 non-SRB (Table 1), were spotted on Hybond-N membrane filters (5 by 5 cm). Up to 80 filters were prepared in one batch. Spotting of 2-μl volumes and covalent linkage by UV irradiation were performed as described before (20). Filters were also dried at 80°C for 10 min prior to irradiation. The amounts of DNA spotted were up to 70-fold larger than in earlier work, in which 10- to 20-ng quantities were applied (20, 21). We were unable to spot the same amount of denatured DNA for each standard, because widely different quantities were available for master filter preparation. The maximum amount of available stock DNA spotted was 0.1%. The actual amounts are given in Table 1. The standards *Lac*1,2, *Lac*3, *Lac*4, *Lac*5, *Lac*6, *Lac*10, *Eth*3, *Ben*1, *Dec*1, *Pro*12, *Ace*1, *Ace*3, and *Ace*4 listed in Table 1 have been described before (20, 21). They all belonged to the saline SRB community previously identified in the WW and WM fields (20). Standards *Lac*24, *Lac*25, and *Lac*26 were obtained as liquid enrichment cultures of samples obtained from unit WW13 on lactate-containing Pfennig's medium. They have been characterized with techniques similar to those described before (20), by hybridizing Southern blots of their *Eco*RI-digested DNAs with both the *Desulfovibrio*-specific [NiFe] hydrogenase gene probe and the general 16S rRNA probe (not shown). Positive hybridization with the former identified them as *Desulfovibrio* spp. (18). A number of non-SRB standards were isolated as single colonies, growing anaerobically on saline tryptone-yeast extract plates or saline minimal glucose plates (as described in references 19 and 8, respectively, but containing 30 g of NaCl per liter). The standards *Sty*1, *Sty*2,3 (a mixture of strongly cross-hybridizing isolates *Sty*2 and *Sty*3), *Sty*4, and *Smg*1 were similarly characterized by hybridizaation with the general 16S rRNA probe. *Sty*2,3 may

TABLE 1. Characteristics of standards applied to the master filters

Standard (identification no.)[a]	Identification[b]	Status[c]	c_x (ng)[d]
*Lac*1,2 (1)	*Desulfovibrio vulgaris* subsp. *oxamicus*	CP	1,450
*Lac*3 (2)	*Desulfovibrio desulfuricans* G200	CP	1,000
*Lac*4 (3)	*Desulfovibrio* sp.	CP	640
*Lac*5 (4)	*Desulfovibrio* sp.	CP	480
*Lac*6 (5)	*Desulfovibrio* sp.	CP	640
*Lac*10 (6)	*Desulfovibrio* sp.	CP	240
*Lac*24 (7)	*Desulfovibrio* sp.	LC	480
*Lac*25 (8)	*Desulfovibrio* sp.	LC	120
*Lac*26 (9)	*Desulfovibrio* sp.	LC	320
*Eth*3 (10)	*Desulfovibrio* sp.	LC	200
*Ben*1 (11)	NI	LC	80
*Dec*1 (12)	NI	LC	30
*Pro*12 (13)	(*Desulfovibrio multivorans*)	LC	40
*Ace*1 (14)	NI	LC	40
*Ace*3 (15)	*Desulfobacter hydrogenophilus*	CP	80
*Ace*4 (16)	NI	LC	20
*Sty*1 (17)	NI	CP	750
*Sty*2,3 (18)	(*Enterobacteriaceae*)	CP	480
*Smg*2 (19)	NI	CP	400
*Sty*4 (20)	NI	CP	640

[a] The first three letters indicate the medium used for isolation. Standards 1 to 16, SRB isolated on Pfennig's medium (12), containing either lactate, ethanol, benzoate, decanoate, propionate, or acetate as the carbon and energy source; standards 17 to 20, non-SRB isolated on either saline tryptone-yeast extract or saline minimal glucose medium as described in the text.

[b] Standards 1, 2, and 15 are type cultures. Assignment of standards 3 to 10 is based on hybridization with a *Desulfovibrio*-specific [NiFe] hydrogenase probe. Identifications in parentheses are based on unpublished 16S rRNA sequencing (standard 13) or cross-hybridization with chromosomal DNA from *E. coli* (standard 18). NI, not identified.

[c] CP, colony purified; LC, stable liquid culture (21).

[d] Amount spotted on the master filter in a 2-μl volume.

belong to the enteric bacteria in view of the cross-hybridization of its chromosomal DNA with the *Escherichia coli* genome (not shown). Finally, denatured bacteriophage λ DNA was spotted on the master filter in amounts of 20, 50, 100, and 200 ng.

Evaluation of hybridization intensities. The hybridization intensity, I_x, defined as the amount of radioactivity bound to immobilized, denatured chromosomal DNA from standard x after incubation of a filter with a reverse genome probe, was determined by liquid scintillation counting or by film densitometry. For scintillation counting, the filters were cut up into 1-cm^2 squares, which were placed in scintillation vials together with 5 ml of CytoScint scintillation fluid (from ICN) prior to counting with an LKB 1215 Rackbeta liquid scintillation counter. For densitometry, the filters were exposed to Fuji RX medical X-ray film for 24 or 72 h. After film development, the films were scanned with an LKB Ultroscan XL laser densitometer, with care taken to scan through the center of the circular hybridization spots. An optical density (OD) scale of 0 to 3.5 was used throughout. OD peak areas (OD_xs) were determined by cutting and weighing. The OD_x is nonlinearly related to I_x (22), which equals the count rate (cpm_x) multiplied by time (t [in minutes]), $I_x = cpm_x \times t$. A calibration curve was established experimentally by spotting 2-μl volumes containing different amounts of [α-^{32}P] dCTP (10^3 to 10^6 cpm) onto a Hybond-N filter and exposing the filter to X-ray film for 30, 90, 370, and 810 min. After the final exposure, the films were scanned and the filter was cut up into 1-cm^2 squares, which were counted. The data, corrected for ^{32}P decay, defined the calibration curve that was used to convert densitometrically determined OD_x values to I_x values.

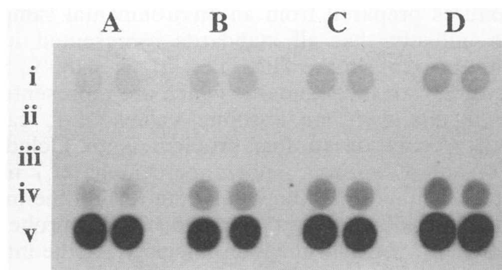


FIG. 1. Reverse gene probing. Duplicate amounts (A, 40 ng; B, 80 ng; C, 120 ng; D, 160 ng) of the following denatured double-stranded or single-stranded DNAs were spotted onto a Hybond-N filter: i, M13mpHV150; ii, M13mp11; iii, pUC9; iv, pHV150; and v, chromosomal DNA from *D. vulgaris* Hildenborough. The last DNA was randomly labeled, and the resulting probe was hybridized with the filter under high-stringency conditions.

An equation for analyzing quantitative RSGP data. When a reverse genome probe is hybridized with a master filter, the I_x, defined as the amount of probe hybridized to immobilized standard x at time t, is (7)

$$I_x = f_x (1 - e^{-k_x' c_x t}) \quad (1)$$

in which f_x is the weight percentage of standard x in the probe, c_x is the weight of chromosomal DNA x spotted on the filter, and k_x' is a hybridization constant dependent on genome complexity. The amount of bound probe increases linearly with time in reverse probe experiments (7) and is a small fraction of total probe added, even for long hybridization times (48 h) and for DNAs of much smaller genome complexity (500 to 1,000 nucleotides) than were considered in this study (10^4 to 10^6 nucleotides). Under these conditions, equation 1 reduces to

$$I_x = (k_x' t) f_x c_x \quad (2)$$

or, for a constant hybridization time,

$$I_x = k_x f_x c_x \quad (3)$$

Sensitivity (for determination of f_x from measured I_x) can thus be raised by increasing c_x. It is for this reason that large amounts of chromosomal DNAs were spotted on master filters used for quantitative RSGP analysis (Table 1). Proportionality between I_x and c_x can easily be shown (Fig. 1). Proportionality between I_x and f_x is expected when, upon labeling of a mixed sample of DNAs, the percentage of label incorporated into a given DNA is proportional to its weight percentage. The DNA polymerase used in the labeling reaction should thus not preferentially allocate label to a given DNA in the preparation. Equation 3 also assumes that there is no cross-hybridization between the chromosomal DNAs of the standards being analyzed. If this assumption is invalid, then I_x will also contain contributions from cross-hybridization.

The validity of equation 3 has been tested in experiments in which mixtures of two or more bacterial standard DNAs were labeled and the resulting probes hybridized with filters containing known, variable amounts of the standard DNAs (not shown). The problem with analyzing more complex

DNA mixtures prepared from an environmental sample is that it is unlikely that all standards represented in the mixture are present on the filter, i.e., Σf_x is <100%. Since the value of Σf_x for the standards which are represented on the filter is unknown, no absolute values of f_x can be calculated. A solution to this problem is to include an internal standard, s, in a known percentage (f_s) in the labeling reaction and in a known amount (c_s) on the master filter. After hybridization of the spiked, labeled probe with the master filter, the hybridization intensity for the internal standard I_s will be

$$I_s = k_s f_s c_s \tag{4}$$

From equations 3 and 4, f_x can be obtained as

$$f_x = (I_x/I_s) \times (k_s/k_x) \times (c_s/c_x) \times f_s \tag{5}$$

We used bacteriophage λ DNA, which does not cross-hybridize with any of the genomic DNAs from the bacterial standards present on the master filter, as the internal standard and used equation 5 for all calculations of f_x from hybridization data.

RESULTS

Feasibility of quantitative RSGP. Since environmental samples may be expected to contain a diversity of bacteria, a preparation of total community DNA will contain different chromosomal DNAs 1, 2,. . . *n*, each present at a weight percentage of f_1, f_2,. . . f_n. Upon random labeling of this mixed DNA preparation, f_x will be the percentage of label allocated to chromosomal DNA fragments of standard *x*. Weak hybridization signals will result if a given bacterial standard is only a minor component of the community. An experiment was designed to prove that $f_x = 0.1\%$ is sufficient for obtaining a detectable hybridization signal. The experiment had the same principle as RSGP but is perhaps more aptly named reverse gene probing. The genes for the [Fe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough have been cloned as a 1.9-kb fragment in vectors pUC9 (15) and M13mp11 (10) in pHV150 and M13mpHV150, respectively (17). When chromosomal DNA of *D. vulgaris* Hildenborough is labeled by random primer extension, approximately 0.1% of the label may be expected to be allocated to this 1.9-kb fragment in view of the size of the *D. vulgaris* genome (~1,700 kb [13]). A filter on which equal weights of (i) the viral positive strand of M13mpHV150, (ii) the viral positive strand of M13mp11, (iii) plasmid vector pUC9, (iv) plasmid pHV150, and (v) chromosomal DNA from *D. vulgaris* Hildenborough had been immobilized was hybridized with the total genome probe. Hybridization of this probe with mpHV150 and pHV150 (Fig. 1, rows i and iv, respectively), as well as the stronger hybridization with chromosomal DNA (Fig. 1, row v), was evident. The DNAs lacking the *D. vulgaris* DNA insert (Fig. 1, rows ii and iii) did not hybridize with the probe. Quantitation by liquid scintillation counting indicated that I_x increased linearly with the amount of DNA spotted (not shown). This establishes that the sensitivity of detection of component *x* can be improved by increasing the amount of denatured, immobilized DNA c_x. For $f_x \approx 0.1\%$, as in the present example, easily detectable hybridization signals were observed for $c_x = 160$ ng (Fig. 1D, row iv). The weaker hybridization of the whole genome probe with the M13mpHV150 DNA, relative to that with the pHV150 DNA, may be caused by the fact that only the viral positive strand was spotted; i.e., f_x is ≈0.05% for this hybridization.

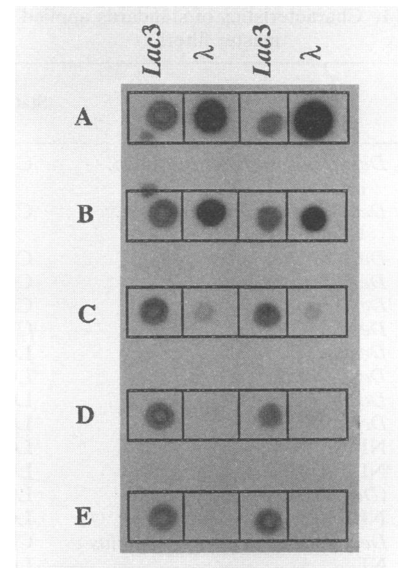


FIG. 2. Reverse genome probing of a mixture of two components of different genomic complexities, *Lac*3 and λ DNA. Each filter contained the following denatured, immobilized DNAs, from left to right: 15.6 ng of *Lac*3, 20 ng of λ, 15.6 ng of *Lac*3, and 20 ng of λ. The filters were hybridized with mixed probes prepared by labeling: A, 78 ng of *Lac*3 and 10 ng of λ; B, 78 ng of *Lac*3 and 1 ng of λ; C, 78 ng of *Lac*3 and 0.1 ng of λ; D, 78 ng of *Lac*3 and 0.01 ng of λ; E, 78 ng of *Lac*3 and 0.001 ng of λ.

Interestingly, the I_x values in row iv were only 10-fold lower than those in row v. Since the amounts spotted were equal, the difference resulted from different label allocations (f_x, 0.1 versus 100%) and from different complexities (k_x, 1.9 versus ~1,700 kb) pertaining to the hybridizations in rows iv and v, respectively.

Determination of k_s/k_x ratio. The influence of genome complexity, which can be assumed to be equivalent to genome size for the purposes of this paper, on the observed hybridization intensity is included in the constant k_x in equation 3. Constant k_x is expected to increase with decreasing genome size as shown in Fig. 2, where mixed whole genome probes consisting of *Lac*3 (~2,000 kb) and bacteriophage λ DNA (48 kb) were hybridized with a filter containing duplicate spots of immobilized, denatured *Lac*3 and λ DNA. When the filter was hybridized with a probe prepared by labeling a mixture of 78 ng of *Lac*3 and 10 ng of λ DNA (Fig. 2A) or 78 ng of *Lac*3 and 1 ng of λ DNA (Fig. 2B), the hybridization intensity with λ (I_λ) exceeded that with *Lac*3 (I_3), despite the fact that *Lac*3 DNA was in considerable excess in both probes. Only when 0.1 ng or less of λ DNA was added to 78 ng of *Lac*3 DNA did hybridization of the resulting mixed genome probe give an I_3 exceeding I_λ (Fig. 2C to E). A number of experiments, as presented in Fig. 2, were performed with bacterial chromosomal DNA from either *Lac*3 or *Lac*6. Possible effects of the purity of the bacterial chromosomal DNA on label allocation were also examined. The results are given in Table 2. The k_λ/k_3 ratios for *Lac*3 DNA (and the standard deviations) were found to be 104 ± 26 for DNA purified with the modified Marmur procedure, 96 ± 16 for DNA purified with an additional CsCl density gradient centrifugation step, and 82 ± 10 for DNA

TABLE 2. Determination of k_λ/k_x ratio

x[a]	Purification[b]	f'_x (ng)[c]	c_x (ng)[d]	I_x[e]	f'_λ (ng)[c]	c_λ (ng)[d]	I_λ[e]	k_λ/k_x[f]
3	(−)	78	15.6	6.7	1	20	12.9	117
3	(−)	78	15.6	15.2	1	20	25.1	100
3	(−)	78	15.6	8.2	2	20	21.5	80
3	(−)	78	15.6	17.3	2	20	38.2	67
3	(−)	78	15.6	7.6	3	20	45.7	122
3	(−)	78	15.6	17.1	3	20	114.0	135
Mean ± SD								104 ± 26
6	(−)	111	22.2	7.0	1	20	10.3	181
6	(−)	111	22.2	16.0	1	20	19.1	147
6	(−)	111	22.2	5.7	2	20	10.9	118
6	(−)	111	22.2	13.0	2	20	20.9	99
6	(−)	111	22.2	7.1	3	20	17.0	98
6	(−)	111	22.2	15.2	3	20	31.8	81
Mean ± SD								121 ± 37
3	CsCl	400	15.6	7.1	1	20	2.6	114
3	CsCl	400	15.6	15.9	1	20	5.7	112
3	CsCl	400	15.6	5.0	2	20	2.6	81
3	CsCl	400	15.6	10.7	2	20	5.1	74
3	CsCl	400	15.6	4.2	3	20	4.1	102
3	CsCl	400	15.6	20.1	3	20	9.2	95
Mean ± SD								96 ± 16
6	CsCl	500	22.2	6.9	1	20	1.8	145
6	CsCl	500	22.2	13.4	1	20	2.8	116
6	CsCl	500	22.2	6.9	2	20	3.4	137
6	CsCl	500	22.2	16.2	2	20	7.1	122
6	CsCl	500	22.2	5.2	3	20	2.0	71
6	CsCl	500	22.2	12.1	3	20	4.6	70
Mean ± SD								110 ± 32
3	Gel	120	15.6	6.7	1	20	5.7	80
3	Gel	120	15.6	14.4	1	20	11.4	74
3	Gel	120	15.6	7.5	2	20	13.8	86
3	Gel	120	15.6	16.2	2	20	25.3	73
3	Gel	120	15.6	7.6	3	20	24.3	100
3	Gel	120	15.6	16.1	3	20	41.3	80
Mean ± SD								82 ± 10
6	Gel	180	22.2	8.1	1	20	4.0	99
6	Gel	180	22.2	15.1	1	20	6.3	83
6	Gel	180	22.2	6.2	2	20	7.0	113
6	Gel	180	22.2	14.5	2	20	14.8	102
6	Gel	180	22.2	4.0	3	20	5.7	95
6	Gel	180	22.2	8.8	3	20	14.5	110
Mean ± SD								100 ± 11

[a] Bacterial chromosomal DNA used; x is either *Lac*3 (shown as 3) or *Lac*6 (shown as 6).
[b] Purification of bacterial chromosomal DNA. (−), Marmur procedure with RNase A and proteinase K digestion; CsCl, same as (−) but with additional RNase A and proteinase K digestion and a CsCl centrifugation purification step; Gel, same as (−) but with additional RNase A and proteinase K digestion and an agarose gel purification step.
[c] f'_x and f'_λ are the total amounts of DNAs x and λ in the labeling reaction. The fractions f_x and f_λ can be calculated as $f_x = [f'_x/(f'_x + f'_\lambda)]$ and $f_\lambda = [f'_\lambda/(f'_x + f'_\lambda)]$. Note: $f'_x/f'_\lambda = f_x/f_\lambda$.
[d] c_x or c_λ, amount of denatured bacterial, chromosomal, or bacteriophage λ DNA spotted on the master filter.
[e] I_x or I_λ values are expressed as relative units obtained by converting OD values by using the OD-versus-I calibration curve.
[f] From equation 5, $k_\lambda/k_x = (I_\lambda/I_x) \times (c_x/c_\lambda) \times (f_x/f_\lambda)$.

prepared with an additional HGT agarose purification step (Table 2). These values are not significantly different, and an average of $k_\lambda/k_3 = 94 \pm 20$ can be calculated. For *Lac*6 DNA, the ratios were 121 ± 37, 110 ± 32, and 100 ± 11, respectively. These values are also the same within the margin of experimental error, giving an average of $k_\lambda/k_6 = 110 \pm 29$. The similarity of k_λ/k_3 and k_λ/k_6 indicates that the genomes of *Lac*3 and *Lac*6 are of similar sizes. Combining all data, an average of $k_\lambda/k_x = 102 \pm 26$ is obtained, and this factor was used throughout for calculation of f_x for bacterial standards in quantitative RSGP experiments in which λ DNA was used as the internal standard. In most of these experiments, 100 ng of total community DNA was spiked with 100 pg of the internal standard ($f_\lambda = 0.1\%$). Under these conditions, equation 5 changes to

$$f_x = 10.2 \times (I_x/I_\lambda) \times (c_\lambda/c_x) = 10.2 \times (I_x/c_x)/(I_\lambda/c_\lambda) \quad (6)$$

DNA isolations. Sixty-eight production water samples (1 liter each) were centrifuged and the pelleted solids were resuspended in 1 ml of 0.15 M NaCl–0.1 M EDTA (pH 8). When 10-μl aliquots of these 1-ml stock solutions were

TABLE 3. Survey of DNAs isolated from production waters and corrosion coupons

Site	DNA concn (μg/liter) in planktonic samples at[a]:				DNA concn (ng/cm²) in biofilm samples[b]
	Wk 1	Wk 3	Wk 5	Wk 7	
WW1					
FWKO	6	0	0	0	0
WP	30	30	50	37	2.5
WW5					
FWKO	0	1	3	0	18
WP	0	0	20	7	380, 127
WW6					
FWKO	3	2	13	4	42, 56
WP	4	3	47	19	190, 282, 106
WW13					
FWKO	NS[c]	NS	NS	NS	0
WP	NS	NS	NS	NS	88
WW14					
FWKO	15	17	3	<0.1	0
WP	50	40	27	19	64, 32
WW20					
FWKO	0	1	0.4	17	17
WP	0	0.3	3	7	42
WW28					
FWKO	0	1	0	0	42
WP	1	0	3	0	0
WWTP					
FWKO	47	17	3	<0.1	0
WP	12	17	<0.1	30	74
WM					
FWKO	0	3	36	<0.1	137
UWP	2	13	47	15	38
LWP	3	13	47	12	46

[a] DNA concentration in production water sample as determined by agarose gel electrophoresis. <0.1, no DNA detected by electrophoresis, but some could be purified; 0, no DNA detected by electrophoresis, and none could be purified.

[b] Amount of DNA purified from metal plugs. 0, no DNA detected after purification. Multiple numbers for a given site represent purifications from different plugs obtained from that site.

[c] NS, not sampled.

electrophoresed through HGT agarose, 1- to 500-ng amounts of DNA were recorded for 49 samples (Table 3), whereas the 10-μl aliquots from 19 stock solutions had DNA concentrations below the detection limit (1 ng). Small amounts of DNA could be purified from 4 of these 19 stock solutions (<0.1 μg/liter [Table 3]), whereas no DNA could be purified from the remainder. Electrophoresis indicated that 80 to 100% of the nucleic acids in the stock solutions migrated into the gel, with the balance remaining in the wells. Apparently the osmotic shock given upon suspending cells from their saline environment into NaCl-EDTA resulted in cell lysis. Some of the production water samples had residues of oil floating on top or suspended particles that could not be recovered by centrifugation. The total DNA prepared from these samples did not include bacteria that might have adhered to these fractions. The DNA concentrations reported for planktonic samples in Table 3 thus represent total DNA of the bacterial fraction that could be pelleted. In general, DNA concentrations in the WPs were higher than those in the FWKOs. This indicates that significant growth must have occurred in the WPs, since flow was from the field to the FWKO and then to the WP. The DNA concentration of the production waters appeared to peak in week 5 in all units, except WW14 and WWTP. If all planktonic DNAs were of bacterial origin and assuming there are 2×10^5 bacteria per ng of DNA, it appears that 10^5 to 10^7 bacteria per ml were present in most production waters.

The biofilm DNAs could be detected only after complete purification. The actual DNA concentrations could thus be significantly higher than the values shown in Table 3, depending on the yield of the purification procedure. Some perspective on these numbers can be gained by considering that (i) if individual bacteria occupied a surface area of ~3 μm^2 and if there were 2×10^5 bacteria per ng of DNA, then a monolayer would maximally yield ~160 ng/cm² and that (ii) scanning electron micrographs of biofilms generated in this system showed that the cells are much more widely separated by an iron sulfide-rich matrix (5). Multiple biofilm DNA isolations were done for sites WW5 WP, WW6 FWKO, WW6 WP, and WW14 WP. Because different starting material was used, the different numbers could be caused by variations in yield or by an actual difference in the thickness of the biofilm or cell density in the biofilm. FWKO biofilm samples often did not yield DNA (four out of nine samples) and tended to give smaller amounts than the WP biofilm samples. The averages for all samples were 31 and 105 ng/cm², respectively. This trend corresponds to that observed for the planktonic phase, in which DNA concentrations were also generally lower in the FWKOs than in the WPs.

Generation of RSGP fingerprints. All 72 DNA preparations listed in Table 3 were analyzed by the RSGP without growth method. As an example, the autoradiograms in Fig. 3A and B represent 24- and 72-h exposures obtained for DNA preparation WM LWP at week 5. The OD_xs for the two exposures (Table 4) were first converted into the corresponding I_x and then divided by c_x, the amount of DNA spotted. The I_λ/c_λ ratio data for the internal standard were averaged, and the average values were used to calculate f_x for all standards (1 to 20) for the 24- and 72-h exposures with equation 6. Good reproducibility of f_x (average deviation of 9% from the mean) was obtained for standards 1 to 11 and 17 to 20. For standards 12 to 16, film blackening after 24 h of exposure was insufficient for OD_x determination. Hybridization of reverse genome probes to these standards was often very weak, which was partly due to the small amounts of DNA spotted on the filter. The data obtained for standards 12 to 16 were therefore omitted from further analysis. The average f_1 to f_{11} and f_{17} to f_{20} values are displayed in a bar diagram (Fig. 3C). It was not necessary to rigorously control autoradiography times for analysis as done for Table 4. Duplication of the entire procedure (including probe preparation, hybridization, autoradiography, and densitometric analysis) gave f_x values that deviated from the mean by an average of 20%. The occasional presence of contaminating hot spots (see arrows in Fig. 3B) prevented reliable determination of I_x by liquid scintillation counting, and this was the main reason for which use of film densitometry was preferred.

RSGP fingerprinting of microbial communities. The planktonic microbial community at WM LWP in week 5 had relatively high levels (up to 1.5%) of non-SRB standards 17 to 20, which together comprised 4.4% of the DNA in the preparation (Fig. 3C). The most prominent SRB was standard 11 (*Ben*1, f_{11} = 1.1%). SRB standards 1 to 11 accounted for 5.4% of the total DNA. Together, the 15 standards thus represented 9.8% of the total DNA, indicating that the master filter used for the analysis provided only partial coverage of the community at WM LWP. The calculated coverage of the community DNA ranged from 1 to 40% for planktonic samples, with an average of 9% (5% SRB and 4%

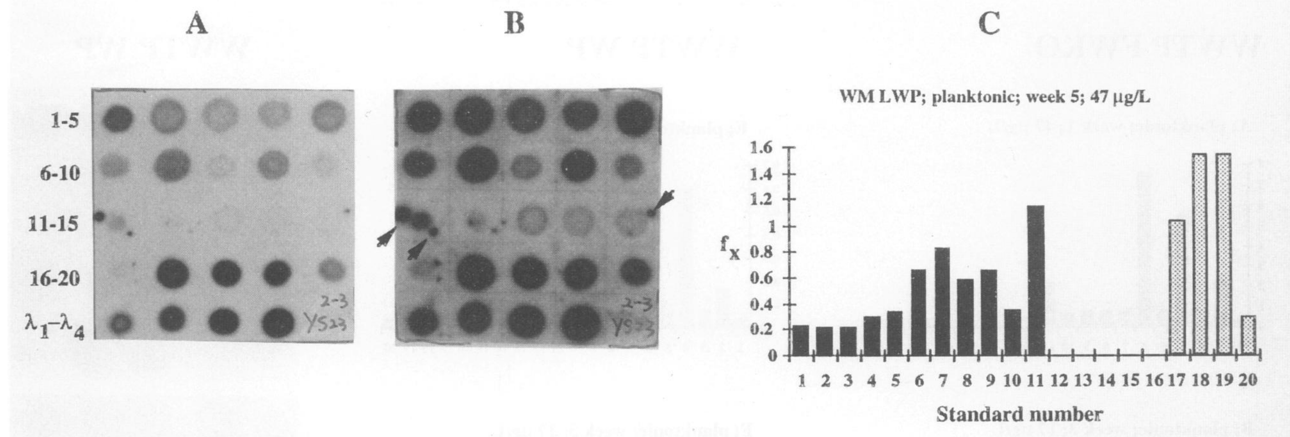


FIG. 3. Quantitative RSGP of DNA prepared for WM LWP, week 5. The labeled reverse genome probe (100 ng of community DNA and 100 pg of λ DNA) was hybridized to the master filter. Autoradiography results for 24 (A) or 72 (B) h, together with the corresponding bar diagram (C), are shown. Generation of f_x values from densitometric data is indicated in Table 4. The standard numbers correspond to those in Tables 1 and 4; SRB (standards 1 to 11) are shaded black, while non-SRB (standards 17 to 20) are shaded gray. The arrows in panel B indicate several contaminating hot spots.

non-SRB). For biofilm samples, the calculated coverage ranged from 3 to 69%, with an average of 21% (18% SRB and 3% non-SRB). In the sections presented below, the term "microbial community" refers to this limited fraction of the total represented on the master filter. It should also be reiterated that biofilm samples were obtained less regularly than planktonic samples and were obtained during a different time period. Therefore, no dates are indicated with the bar diagrams for the biofilm samples in Fig. 4 to 7, even when multiple samples were obtained and analyzed (Fig. 5 and 7).

A summary of all bar diagrams obtained for planktonic and biofilm samples from the WWTP is shown in Fig. 4. The planktonic community fingerprints at week 1 for the FWKO (Fig. 4A) and WP (Fig. 4E) were very similar, showing a relatively high percentage of standards 4, 6, and 11 (*Lac*5, *Lac*10, and *Ben*1). This same feature is also apparent in the diagrams obtained for weeks 3 and 5 (Fig. 4B, C, F, and G). At week 7, the planktonic FWKO community was dominated by standard 6 (*Lac*10, f_6 = 6%) and the WP community was dominated by standard 11 (*Ben*1, f_{11} = 7%). The

TABLE 4. Data from quantitative RSGP analysis of autoradiograms shown in Fig. 3A and B

Standard (identification no.)	c_x (ng)	~72-h exposure				~24-h exposure				f_x (avg)[c]
		OD_x	I_x	I_x/c_x[a]	f_x	OD_x	I_x	I_x/c_x[b]	f_x	
*Lac*1,2 (1)	1,450	79.2	72.4	0.0499	0.201	36.1	30.1	0.0208	0.253	0.227
*Lac*3 (2)	1,000	66.8	53.2	0.0532	0.214	16.2	16.8	0.0168	0.205	0.209
*Lac*4 (3)	640	45.6	36.2	0.0566	0.227	8.2	10.0	0.0156	0.190	0.209
*Lac*5 (4)	480	45.9	36.4	0.0758	0.305	9.0	10.8	0.0225	0.274	0.290
*Lac*6 (5)	640	70.3	57.5	0.0898	0.361	16.4	16.9	0.0264	0.322	0.342
*Lac*10 (6)	240	49.9	39.0	0.1625	0.653	11.1	12.6	0.0525	0.640	0.647
*Lac*24 (7)	480	96.4	118.4	0.2467	0.992	29.1	25.4	0.0529	0.645	0.818
*Lac*25 (8)	120	20.3	19.6	0.1633	0.657	3.3	5.0	0.0417	0.508	0.582
*Lac*26 (9)	320	66.4	52.6	0.1644	0.661	16.1	16.7	0.0522	0.636	0.648
*Eth*3 (10)	200	20.9	20.0	0.1000	0.402	3.2	4.8	0.0240	0.292	0.347
*Ben*1 (11)	80	22.2	20.9	0.2613	1.050	5.8	7.8	0.0975	1.188	1.119
*Dec*1 (12)	30	7.5	9.3	0.3100	1.246					
*Pro*12 (13)	40	11.4	12.9	0.3225	1.297					
*Ace*1 (14)	40	8.9	10.7	0.2675	1.075					
*Ace*3 (15)	80	6.7	8.6	0.1075	0.432					
*Ace*4 (16)	20	16.0	16.6	0.8300	3.337					
*Sty*1 (17)	750	118.4	231.6	0.3088	1.242	63.2	48.2	0.0643	0.783	1.012
*Sty*2,3 (18)	480	115.4	208.1	0.4335	1.743	65.5	51.3	0.1069	1.302	1.523
*Smg*2 (19)	400	106.0	157.0	0.3925	1.578	63.3	48.3	0.1208	1.472	1.525
*Sty*4 (20)	640	56.4	43.3	0.0677	0.272	15.5	16.2	0.0253	0.308	0.290
λ1	20	64.6	49.9	2.4950		24.4	22.4	1.1200		
λ2	50	98.4	125.4	2.5080		45.6	36.2	0.7240		
λ3	100	122.3	268.5	2.6850		79.5	73.0	0.7300		
λ4	200	138.6	492.0	2.4600		105.5	154.8	0.7740		

[a] The average for λ1 to λ4 values was 2.5370.
[b] The average for λ1 to λ4 values was 0.8470.
[c] These data are represented by the bar diagram in Fig. 3C.

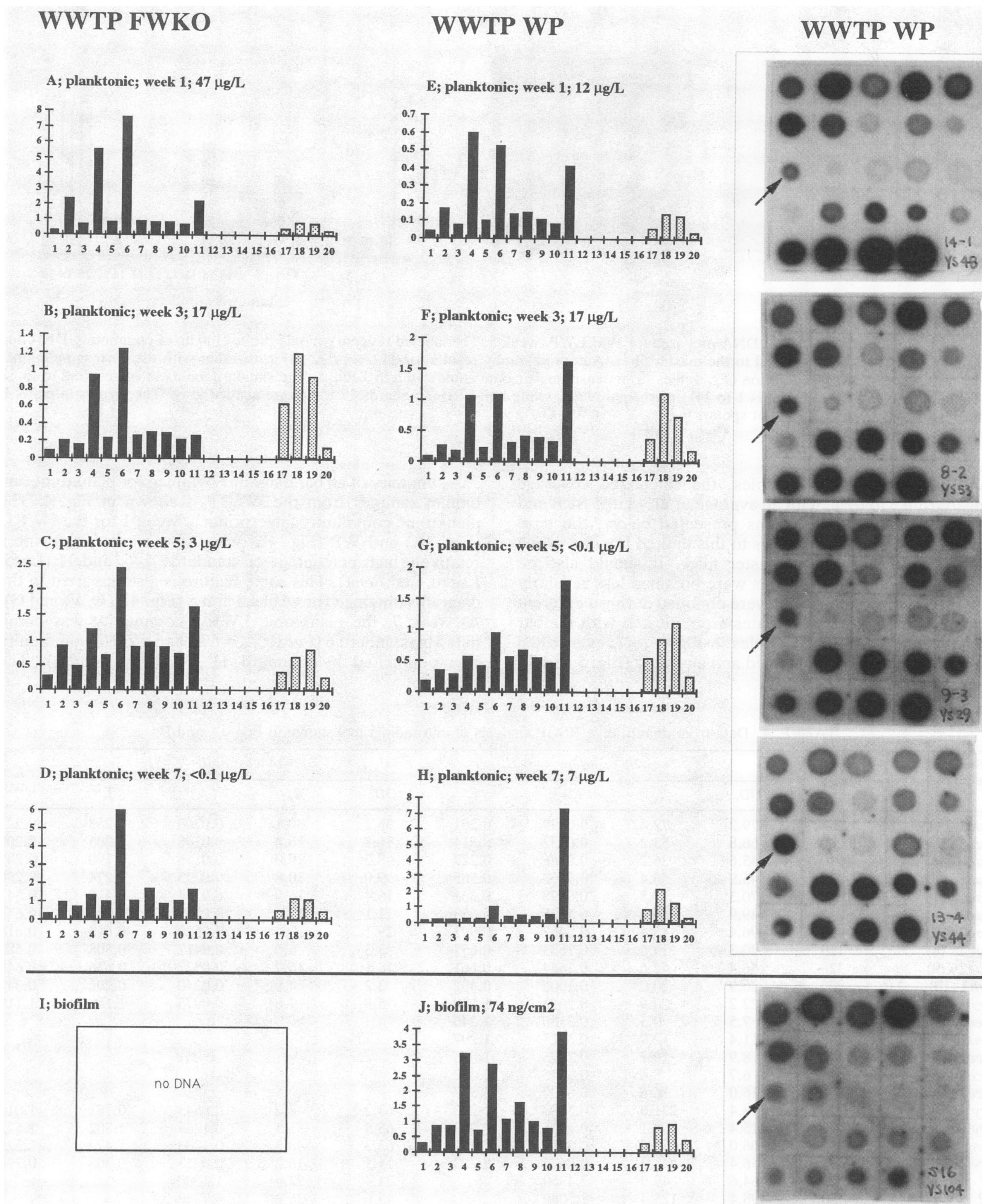


FIG. 4. Survey of all RSGP bar diagrams obtained for samples from the WWTP FWKO and WWTP WP. The meaning of the axes in each diagram is the same as in Fig. 3C. The autoradiograms (72-h exposure) corresponding to the WWTP WP bar diagrams are also shown. Arrows indicate hybridization to standard 11 (*Ben*1). Results for planktonic (A to H) and biofilm (I to J) samples are separated by the horizontal bar. Biofilm samples are not dated for reasons described in the text.

increase in *Ben*1 in the planktonic WWTP WP samples with time is also evident from the autoradiograms (Fig. 4, arrows). A WP biofilm sample (Fig. 4J) showed a community profile very similar to the WP planktonic profiles in weeks 1 and 3 (Fig. 4E and F).

In Fig. 5, the planktonic community fingerprints in weeks 1 and 7 for site WW6 in the WW field are shown together with all of the results obtained for biofilm samples. Significant changes in the planktonic community are seen in going from the FWKO to the WP: standard 10 (*Eth*3) increased significantly, in both weeks 1 and 7, whereas the non-SRB (standards 17 to 20) showed a significant decrease. Biofilm samples from WW6 WP (Fig. 5G to I) showed high percentages of *Eth*3, as well as *Lac*6 and *Lac*24 (standards 5 and 7). *Eth*3 comprised 33% of the isolated biofilm DNA in Fig. 5G and 7% in Fig. 5H. SRB standards other than 5, 7, and 10, as well as non-SRB standards 17 to 20, were present as only minor percentages of the biofilm communities in Fig. 5G and H. The biofilm sample shown in Fig. 5I consisted of a more evenly distributed community in which SRB standards 5, 7, and 10 were still dominant. The same applies to the FWKO biofilm communities, which showed relatively high levels of standard 8 (*Lac*25 [Fig. 5E]) or standard 10 (*Eth*3 [Fig. 5F]). The percentages calculated for these more evenly distributed biofilm communities are similar to those calculated for planktonic samples. Dominance of *Eth*3 in WP samples is a feature typical of unit WW6 (Fig. 5, WW6 WP, arrows) and was not found in any of the other production units studied (also see Fig. 4, 6, and 7).

The bar diagrams in Fig. 6 present an example of a stable microbial community in the WM field. Little or no change in community fingerprints was seen when production waters which flowed from the FWKO to the UWP and then to the LWP were analyzed in either week 1 (Fig. 6E and I), week 3 (Fig. 6B, F, and J), week 5 (Fig. 6C, G, and K), or week 7 (Fig. 6D, H, and L). The planktonic SRB community was dominated by standard 11 (*Ben*1), with frequent peaks at standards 6 and 8 (*Lac*10 and *Lac*25). The WW14 WP biofilm communities show elevated percentages of standard 6 (Fig. 6N and O [5 and 11%, respectively]). *Ben*1 and non-SRB are present at much lower levels. The FWKO biofilm sample (Fig. 6M) again indicates a more evenly distributed community.

Finally, results for two WP communities, WW14 WP and WW5 WP, are shown in Fig. 7. The planktonic communities in week 5 (Fig. 7A and C) were similar and resembled those of the WM field (Fig. 6). The planktonic WW5 WP community at week 7 had an elevated level of standard 7 (*Lac*24). That standard strongly predominated in biofilm samples from WW5 WP (Fig. 7G and H [27 and 13%, respectively]). Hybridization of standard 7 with labeled community DNA prepared from WW5 WP samples has been highlighted in the autoradiograms (Fig. 7, WW5 WP, arrows). The WW14 WP biofilms showed the dominance of *Lac*24 in a more evenly distributed community (Fig. 7E and F), and a similar result was obtained for a WW5 FWKO biofilm (not shown).

DISCUSSION

In the present paper, we have shown that quantitative RSGP can be a useful tool for fingerprinting microbial communities in the environment. The chosen environment, saline oil field production waters and the metal surfaces in contact with these waters, had the advantage that total community DNA was obtained rather easily. We found that the RSGP fingerprints obtained for samples from different sites from this environment were highly diagnostic. This was already clear prior to the completion of quantitative analysis of the autoradiograms. When various autoradiograms (Fig. 3 to 5 and 7) were examined, diagnostic features were evident even before quantitative analysis had been completed; e.g., those belonging to the WWTP or WW6 were easily sorted out.

There are several potential sources of error, which may cause the calculated f_x to deviate from the actual values in the sample. (i) It is important that the DNA preparation is representative of the bacteria in the sample. The method used here was originally designed to isolate high-molecular-weight double-stranded DNA (9, 18). Although this method appeared adequate, it may not be the best method for DNA isolations from other environments. Much more rigorous conditions can be used to achieve cell lysis (e.g., alkali treatment or sonication) since, in principle, a representative single-stranded DNA preparation of reasonable average length is all that is required for the analysis. (ii) Another concern was that the DNA preparations obtained might contain inhibitors of the labeling reaction. Two possibilities should be distinguished: (a) the presence of reversible polymerase inhibitors affecting the labeling of all DNA molecules in the preparation (including the added internal standard) equally and (b) the presence of irreversibly bound inhibitors (e.g., residual proteins) affecting the labeling differentially. With respect to possibility a, we have consistently observed that the internal standard was labeled less intensely when mixed with biofilm DNA than when mixed with planktonic DNA (Fig. 4, 5, and 7). The source of this apparent inhibition of the labeling reaction must be the biofilm DNA, and we assume that it affects all DNAs equally. With respect to possibility b, it appeared that when either of three grades of bacterial chromosomal DNA from *Lac*3 or *Lac*6 was labeled together with added λ DNA, the label allocation was the same irrespective of DNA quality or source (Table 2), indicating that differential, irreversible inhibition was not affecting the labeling of these chromosomal DNAs. If this is important in the labeling of mixtures of total community DNA and the internal standard, it would lead to an underestimation of f_x. (iii) The calculation of f_x has assumed that all bacterial standards have the same genome complexity. This assumption has been proven to be reasonable for *Lac*3 and *Lac*6 but may not hold for bacterial standards that have a genome either significantly smaller or significantly larger than those of these two *Desulfovibrio* species. In principle, the k_λ/k_x ratio can be determined for every standard on the filter and the values determined can be used to calculate a more accurate f_x. Knowledge of the dependence of the k_λ/k_x ratio on genome size would allow various environmental DNAs (including plasmids and those from viruses) to be simultaneously analyzed by quantitative RSGP. (iv) It is clear from equations 5 and 6 that errors in determination of the concentration of DNA will directly affect the calculated f_x values; the dependence of I_x on c_x may also not be linear at very high c_x values. Factors i to iv caution against interpretations of the results that rely solely on the absolute values of the derived f_x.

The features of RSGP fingerprints obtained for individual oil field sites (Fig. 4 to 7) have been outlined in the Results section. The finding that these fingerprints sometimes differed significantly was surprising, because all units except WWTP processed oil-water mixtures from the same geological formation. Comparison of fingerprints for planktonic and biofilm samples from the same site reveals some interesting biofilm properties. SRB play an important role in the corro-

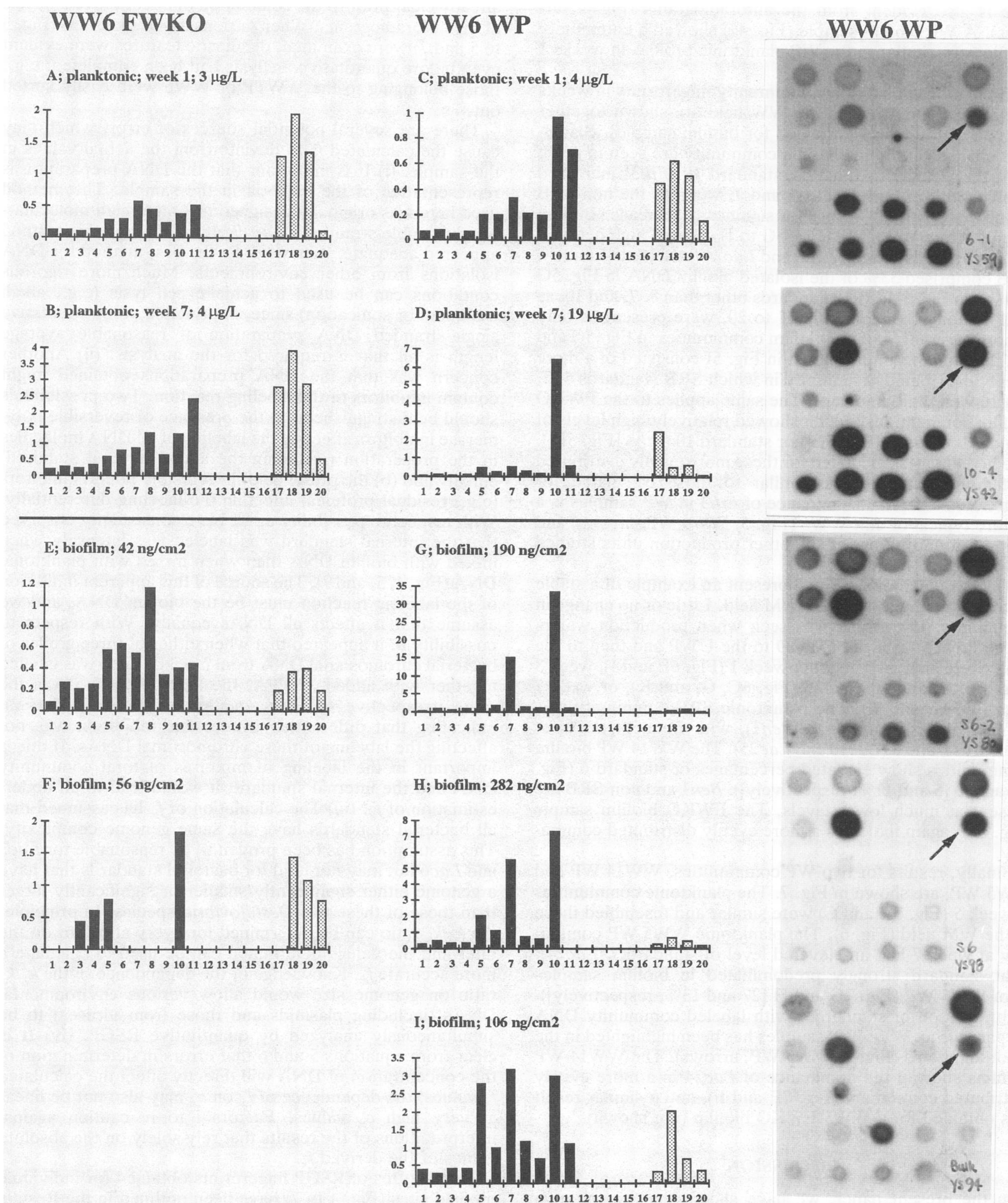
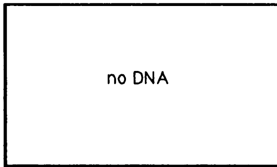


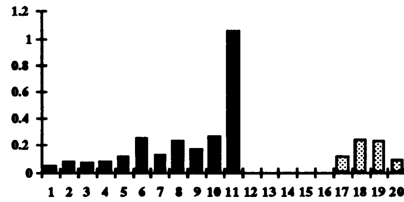
FIG. 5. Survey of RSGP bar diagrams obtained for samples from the WW6 FWKO and WW6 WP. The meaning of the axes in each diagram is the same as in Fig. 3C. The autoradiograms (72-h exposure) corresponding to the WW6 WP bar diagrams are also shown. Arrows indicate hybridization to standard 10 (*Eth*3). Results for planktonic samples (A to D, weeks 1 and 7) and biofilm samples (E to I) are separated by the horizontal bar. Biofilm samples are not dated for reasons described in the text.

WM FWKO **WM UWP** **WM LWP**

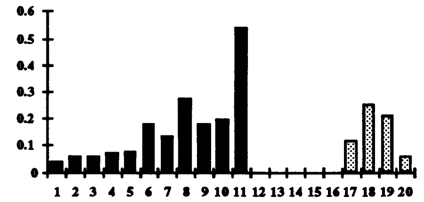
A; planktonic; week 1; 0 μg/L



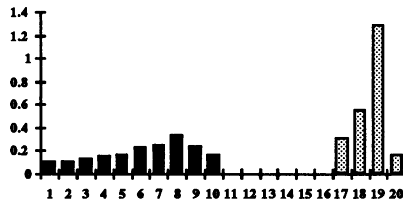
E; planktonic; week 1; 2 μg/L



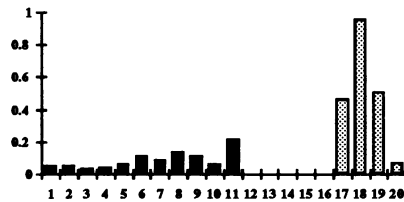
I ; planktonic; week 1; 3 μg/L



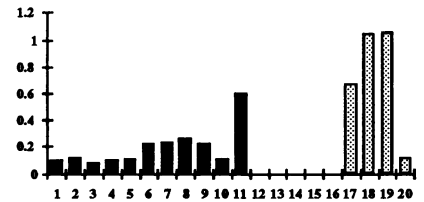
B; planktonic; week 3; 3 μg/L



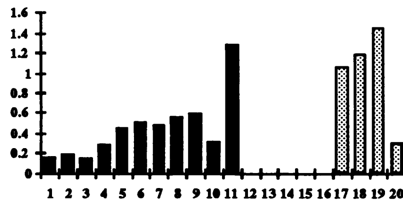
F; planktonic; week 3; 13 μg/L



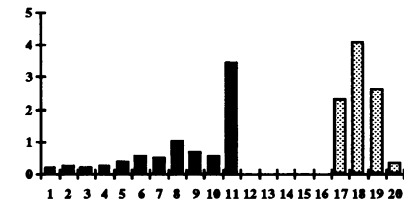
J; planktonic; week 3; 13 μg/L



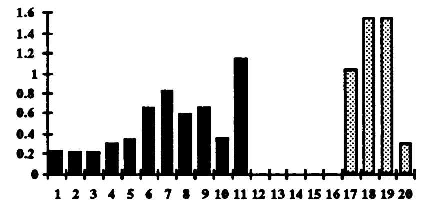
C; planktonic; week 5; 36 μg/L



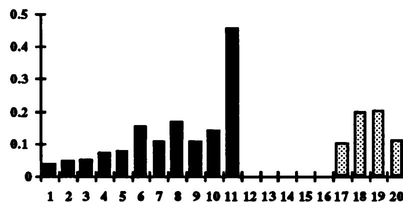
G; planktonic; week 5; 47 μg/L



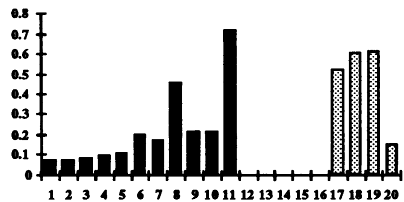
K; planktonic; week 5; 47 μg/L



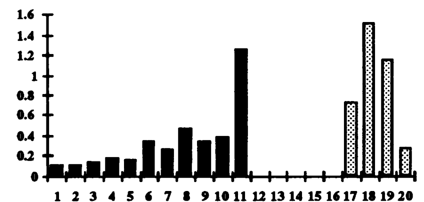
D; planktonic; week 7; <0.1 μg/L



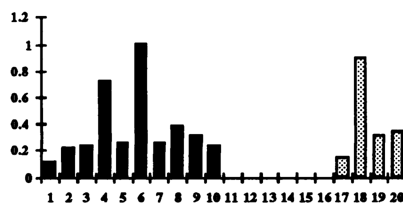
H; planktonic; week 7; 15 μg/L



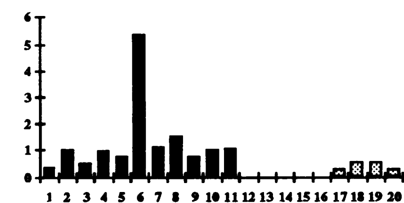
L; planktonic; week 7; 12 μg/L



M; biofilm; 137 ng/cm2



N; biofilm; 38 ng/cm2



O; biofilm; 46 ng/cm2

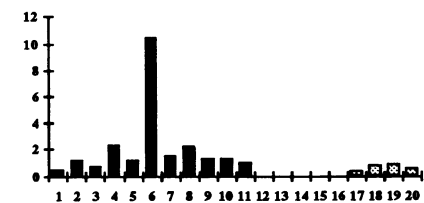


FIG. 6. Survey of all RSGP bar diagrams obtained for samples from the WM FWKO, WM UWP, and WM LWP. The meaning of the axes in each diagram is the same as in Fig. 3C. Results for planktonic (A to L) and biofilm (M to O) samples are separated by the horizontal bar. Biofilm samples are not dated for reasons described in the text.

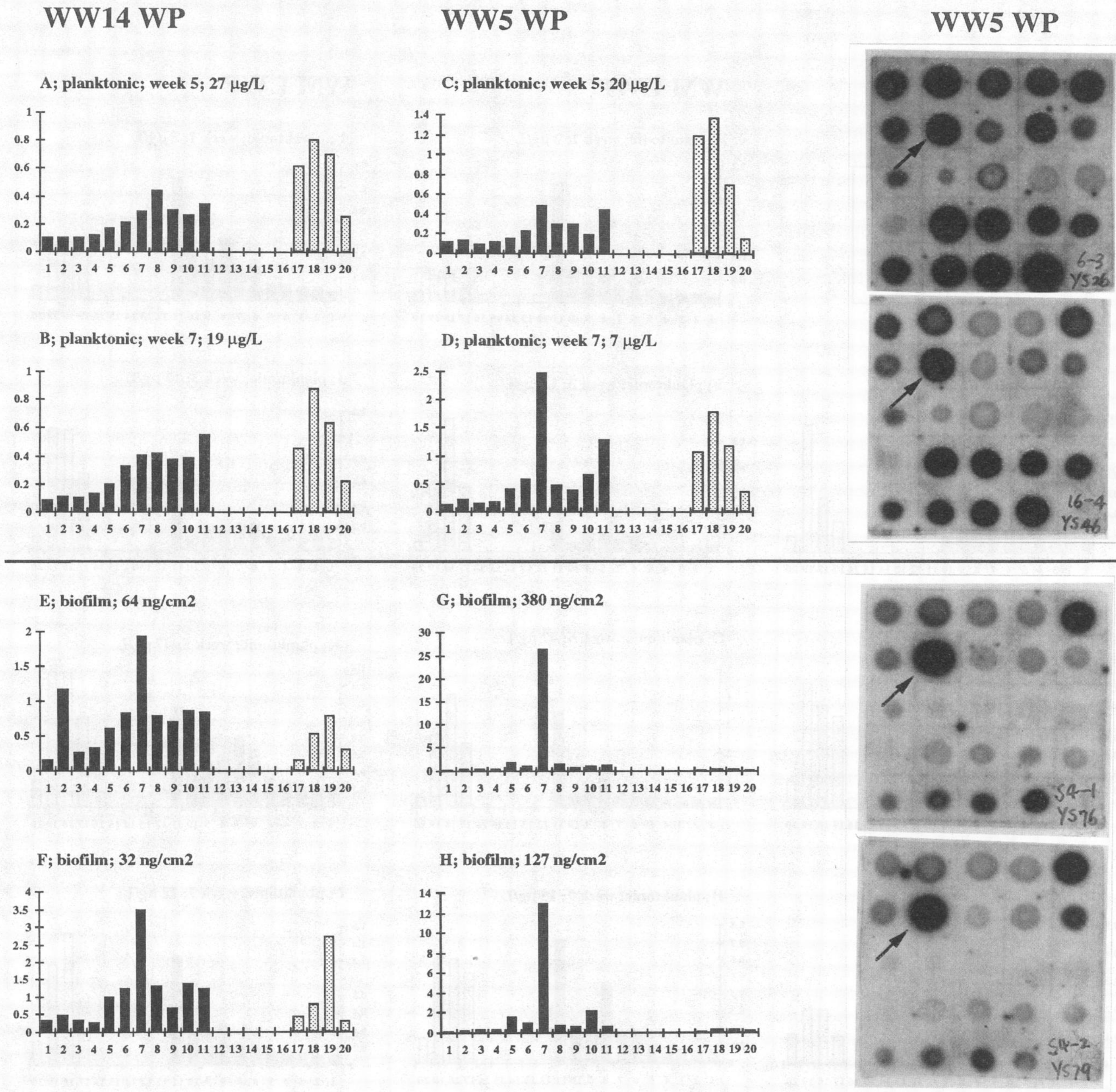


FIG. 7. Survey of some of the RSGP bar diagrams obtained for samples from the WW14 WP and WW5 WP. The meaning of the axes in each diagram is the same as in Fig. 3C. The autoradiograms (72-h exposure) corresponding to the WW5 WP bar diagrams are also shown. Arrows indicate hybridization to standard 7 (*Lac*24). Results for planktonic (A to D) and biofilm (E to H) samples are separated by the horizontal bar. Biofilm samples are not dated for reasons described in the text.

sion of metal (6). A possible mechanism for the SRB-mediated dissolution of iron under anaerobic conditions was first formulated by von Wolzogen Kühr and van der Vlugt (16). In this mechanism, SRB catalyze the anaerobic dissolution of iron by metabolic oxidation of cathodic hydrogen from the metal surface and formation of iron sulfide as a corrosion product (6, 11). A refined model, in which the produced iron sulfide acts as the depolarizing agent that mediates electron or hydrogen transfer from the metal surface to the SRB, has been presented by Tiller (14). Evidence for this mechanism was provided by (i) the proven depolarizing properties of iron sulfide, (ii) the observation that hydrogenase-negative SRB can also be effective in metal corrosion, and (iii) the fact that bacteria in corroding biofilms are located in an iron-sulfide-rich matrix at the biofilm-fluid interface, not at the biofilm-metal interface (5). Both mechanisms are discussed in depth by Hamilton (6).

Work by Pfennig et al. (12) and classification by 16S rRNA sequencing (2) have indicated that the SRB consist of at least eight genera. SRB from the genus *Desulfovibrio*, which is very diverse (3), are easily isolated from the environment and have been shown to always contain at least one periplas-

mic hydrogenase (18). *Desulfovibrio* spp. could thus contribute to metal corrosion through either or both of the mechanisms described above. Laboratory studies have indicated that both hydrogenase-positive *Desulfovibrio* species (11) and hydrogenase-negative SRB of other genera (6, 11) are capable of causing metal weight loss by themselves. A consortium of three bacteria, a *Desulfovibrio* sp., a *Desulfobacter* sp., and *Eubacterium limosum*, was shown to be more effective than combinations of two or single strains (4). Scanning electron micrographs indicated that a *Desulfovibrio* sp. predominated at the metal surface upon incubation with an *E. limosum-Desulfovibrio* coculture. Identification of SRB in multispecies biofilms was also accomplished by visualization of bacteria by fluorescence microscopy after staining with a specific fluorescent 16S rRNA probe (1).

The question left unanswered by these laboratory studies is which bacteria predominate in metal-associated biofilms in natural systems. We have shown here that RSGP without growth provides an approach to answer this question. Of the 20 standards spotted on the master filter, 10 (standards 1 to 10) were *Desulfovibrio* species, whereas 6 (standards 11 to 16) were non-*Desulfovibrio* SRB (Table 1). One of these, *Ben*1, which was frequently obtained as a liquid culture enrichment from saline oil field samples (21), appeared to be present to a high percentage in many planktonic communities (e.g., Fig. 4E to H and Fig. 6). Nevertheless, *Ben*1 did not appear as a major component in many biofilm samples (Fig. 5G to I, Fig. 6N and O, and Fig. 7G and H). Similarly, although we did not present a quantitative analysis for the presence of the five other non-*Desulfovibrio* SRB (standards 12 to 16, *Dec*1, *Pro*12, *Ace*1, *Ace*3, and *Ace*4, respectively) qualitative comparison of I_x for planktonic and biofilm samples does not indicate these to be increased in the biofilm community (e.g., compare the autoradiograms for Fig. 7C and D with those for Fig. 7G and H). The non-SRB standards 17 to 20 were also not increased in the biofilm population. In contrast, *Desulfovibrio* species (standards 1 to 10) can be strongly enhanced in the metal-associated biofilm, with some standards reaching a calculated 5 to 30% of the DNA isolated from the community. It is interesting that only some of the 10 *Desulfovibrio* standards spotted on the master filter (*Lac*6, *Lac*10, *Lac*24, and *Eth*3) were found to have the potential of strongly enhanced growth in the metal-associated biofilm.

Review of all RSGP fingerprints for biofilm samples allows two types of communities to be distinguished. One (type A) resembles the planktonic phase in diversity (Fig. 4J; Fig. 5E, F, and I; Fig. 6M; and Fig. 7E and F), while a second one (type B) is characterized by the dominance of selected *Desulfovibrio* species (Fig. 5G and H, Fig. 6N and O, and Fig. 7G and H). Type B was not found in the FWKOs. This could be caused by the higher temperatures in these units, which may foster surface growth of bacterial standards that are not represented on the master filter; all 20 standards were cultured at 22 to 35°C, a temperature regimen found in the field and in the WPs. Establishment of a biofilm with large fractions of these mesophiles may be prevented by the elevated temperatures in the FWKOs. Biofilm formation in the WPs may occur in two stages, in which (i) general attachment of planktonic cells to newly installed metal plugs leads to the establishment of a diverse biofilm with a community structure reminiscent of the planktonic phase (type A) and (ii) further development of the community allows selective SRB to be greatly enhanced and to form a less-diverse biofilm (type B). Formation of a type B biofilm may depend on water chemistry, which has been shown to be variable for the various sites studied. Regular observation of type B biofilm establishment within the 4-week period allowed for biofilm formation may indicate a strong potential for anaerobic microbial corrosion. Of all units in the WW and WM fields, WW5 WP and WW6 WP were considered to have the most problems in this regard, and this may correlate with the regular observation of type B biofilm establishment (Fig. 5G and H and Fig. 7G and H). A future challenge is therefore to establish the specific properties of *Desulfovibrio* species *Lac*6, *Lac*10, *Lac*24, and *Eth*3 as well as the environmental conditions that allow establishment of type B biofilms on metal surfaces.

ACKNOWLEDGMENTS

This work was supported by a strategic grant of the Natural Science and Engineering Research Council of Canada (NSERC) and by an NSERC Collaborative Research and Development grant with Novacor Research and Technology Corp. C.S.H. was supported by the Natural Environment Research Council.

We are indebted to Ed Rogoz of Husky Oil Ltd. for providing the samples. Phil Fedorak and Julia Foght are thanked for their help in maintaining the SRB standard collection and discussions during the course of this project. The fine technical assistance of Sara Ebert, Rich Keon, Neili Sifeldeen, Johanna K. Voordouw, and Brenda Bramhill is gratefully acknowledged.

REFERENCES

1. **Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:**614–623.
2. **Devereux, R., M. Delaney, F. Widdel, and D. A. Stahl.** 1989. Natural relationships among sulfate-reducing eubacteria. J. Bacteriol. **171:**6689–6695.
3. **Devereux, R., S.-H. He, C. L. Doyle, S. Orkland, D. A. Stahl, J. LeGall, and W. B. Whitman.** 1990. Diversity and origin of *Desulfovibrio* species: phylogenetic definition of a family. J. Bacteriol. **172:**3609–3619.
4. **Dowling, N. J. E., S. A. Brooks, T. J. Phelps, and D. C. White.** 1992. Effects of selection and fate of substrates supplied to anaerobic bacteria involved in the corrosion of pipeline steel. J. Ind. Microbiol. **10:**207–215.
5. **Ferris, F. G., T. R. Jack, and B. J. Bramhill.** 1992. Corrosion products associated with attached bacteria at an oil field water injection plant. Can. J. Microbiol. **38:**1320–1324.
6. **Hamilton, W. A.** 1985. Sulphate-reducing bacteria and anaerobic corrosion. Annu. Rev. Microbiol. **39:**195–217.
7. **Kafatos, F. C., C. W. Jones, and A. Efstratiadis.** 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. **7:**1541–1552.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3:**208–218.
10. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–79.
11. **Pankhania, I. P., A. N. Moosavi, and W. A. Hamilton.** 1986. Utilization of cathodic hydrogen by *Desulfovibrio vulgaris* (Hildenborough). J. Gen. Microbiol. **132:**3357–3365.
12. **Pfennig, N., F. Widdel, and H. G. Truper.** 1981. Dissimilatory sulphate-reducing bacteria, p. 926–940. *In* M. P. Starr (ed.), The prokaryotes, vol. 1. Springer-Verlag, Inc., New York.
13. **Postgate, J. R., H. M. Kent, R. L. Robson, and J. A. Chesshyre.** 1984. The genomes of *Desulfovibrio gigas* and *D. vulgaris*. J. Gen. Microbiol. **130:**1597–1601.
14. **Tiller, A. K.** 1983. Electrochemical aspects of microbial corrosion: an overview, p. 54–65. *In* Microbial corrosion. The Metals Society, London.
15. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7 derived system for insertion mutagenesis and sequencing with

synthetic universal primers. Gene **19:**259–268.

16. **von Wohlzogen Kühr, C. A. H., and L. S. van der Vlugt.** 1934. Graphitization of cast iron as an electrobiochemical process in anaerobic soils. Water **18:**147–165.
17. **Voordouw, G., W. R. Hagen, M. Krüse-Wolters, A. van Berkel-Arts, and C. Veeger.** 1987. Purification and characterization of *Desulfovibrio vulgaris* (Hildenborough) hydrogenase expressed in *Escherichia coli*. Eur. J. Biochem. **162:**31–36.
18. **Voordouw, G., V. Niviere, F. G. Ferris, P. M. Fedorak, and D. W. S. Westlake.** 1990. The distribution of hydrogenase genes in *Desulfovibrio* spp. and their use in identification of species from the oil field environment. Appl. Environ. Microbiol. **56:** 3748–3754.
19. **Voordouw, G., J. D. Strang, and F. R. Wilson.** 1989. Organization of the genes encoding [Fe] hydrogenase in *Desulfovibrio vulgaris* subsp. *oxamicus* Monticello. J. Bacteriol. **171:**3881–3889.
20. **Voordouw, G., J. K. Voordouw, T. R. Jack, J. Foght, P. M. Fedorak, and D. W. S. Westlake.** 1992. Identification of distinct communities of sulfate-reducing bacteria in oil fields by reverse sample genome probing. Appl. Environ. Microbiol. **58:**3542–3552.
21. **Voordouw, G., J. K. Voordouw, R. R. Karkhoff-Schweizer, P. M. Fedorak, and D. W. S. Westlake.** 1991. Reverse sample genome probing, a new technique for identification of bacteria in environmental samples by DNA hybridization, and its application to the identification of sulfate-reducing bacteria in oil field samples. Appl. Environ. Microbiol. **57:**3070–3078.
22. **Wygant, M. D., and J. W. Nelson.** 1993. Improving quantitation in X-ray film autoradiography. Am. Lab. **25:**40RRR–40UUU.