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Detection of *Desulfobacter* in oil field environments by non-radioactive DNA probes

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Abstract Acetate-utilizing, sulfate-reducing bacteria of the genus *Desulfobacter* were enriched from an oilfield seawater-injection system. These bacteria were detected in enrichment culture and verified as *Desulfobacter* by an oligonucleotide DNA probe that targets a *Desulfobacter*-specific sequence of ribosomal RNA. These *Desulfobacter* produced extensive biofilm in culture and exhibited high levels of hydrogenase activity, which suggests a sessile habit and a role in the cathodic depolarization mechanism of microbially influenced corrosion. Phospholipid analyses indicated that one of the oilfield isolates of *Desulfobacter* lacked the fatty acid 10Me16:0, which has been used as a biomarker for this genus. *Desulfobacter* were infrequently encountered in the oilfield systems studied, and they constituted a small portion of the total microflora at any sampling location.

Introduction

Microbial populations are monitored in many oil-production systems because bacteria have been implicated in accelerating corrosion, stabilizing oil/water emulsions, producing biomass that accumulates in production vessels, and contributing to injectivity losses. Sulfate-reducing bacteria (SRB) are considered to be a particular problem because they produce corrosive and toxic H_2S, and historically they have been the main focus of concern in oilfield microbiology.

Monitoring of sulfate-reducing bacteria has focused almost exclusively on *Desulfovibrio* and *Desulfotomaculum*, which have been cultured and characterized for almost 100 years. For many decades these were the only sulfate-reducing bacteria known. Within the last 15 years, however, additional groups of sulfate-reducing bacteria have been discovered, and *Desulfovibrio* and *Desulfotomaculum* are now known to be only two of perhaps ten eubacterial genera of SRB (Widdel 1980; Devereux et al. 1989). The most common method of oilfield bacterial enumeration is by serial dilution, i.e., successive dilution of planktonic or dispersed sessile samples into bacterial culture media. Many oilfield SRB media, however, were formulated for culture of *Desulfovibrio* and *Desulfotomaculum*, and would not be expected to support the growth of the more recently discovered genera of SRB, which have different growth requirements.

Species composition and community structure can be elucidated by molecular methods (Amann et al. 1992), and hybridization assays have revealed considerable diversity in oilfield SRB populations (Voordouw et al. 1993). The oilfield distribution of at least eight genera of SRB is uncertain, however, because of the lack of comprehensive microbiological screening in most oilfield systems. The purpose of the present study was to survey for *Desulfobacter* to determine whether it: (a) occurred in selected oil/water handling systems; (b) contributed significantly to the total SRB population; and (c) had characteristics that could render it particularly destructive.

Selection of a target genus

Sulfate-reducing genera occur in both (eu) bacteria and archaea. Discovery of *Archaeoglobus* (Stetter et al. 1987), which can grow at temperatures over 90°C,

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raised the possibility that sulfate-reducing microorganisms might grow in deep, hot, oil reservoirs; and in very hot parts of oil production systems. Recent results (Stetter et al. 1993) have shown that *Archaeoglobus* and other sulfidogenic archaea are present in co-produced water from oil reservoirs in Alaska and in the North Sea.

Desulfotomaculum is the only gram-positive, eubacterial SRB. This genus is well characterized in the oilfield, and many SRB serial-dilution media will support their growth. *Desulfotomaculum* are relatively ubiquitous, and they are cultured routinely in oilfield microbiology at mesophilic and thermophilic temperatures (approx. 30°C and 55°C).

In 1980, Widdel described several new genera of gram-negative, eubacterial SRB. This had potentially important implications because these bacteria would not grow in standard SRB serial-dilution media, and hence would not be detected by techniques commonly used in oilfield microbiology. Gram-negative genera of SRB that might be present and escape detection include *Desulfobacter*, *Desulfobulbus*, *Desulfococcus*, *Desulfosarcina*, *Desulfonema* and *Desulfobacterium*. The relationships among these genera were elucidated by Devereux et al. (1989), using 16S ribosomal RNA sequence comparisons. Among the gram-negative eubacteria, *Desulfovibrio* is the only SRB that is well characterized in the oilfield.

It is often assumed that *Desulfovibrio* and *Desulfotomaculum* are the predominant SRB because they have been cultured routinely for decades. Now that other SRB are becoming more widely known and studied, however, reports are accumulating that these genera are not always the predominating SRB in natural environments. For example, fatty acid analyses indicated that *Desulfobulbus* and *Desulfobacter* were the most commonly enriched SRB from some sediments (Parkes 1987; Taylor and Parkes 1985). Similar examples have been found to oilfield environments. *Desulfobacter* were found to be the predominant SRB in an oil treater in a German oilfield (Cord-Ruwisch et al. 1985, 1987). Fatty-acid biomarkers indicated low populations of *Desulfovibrio* but high populations of *Desulfobacter* in a Texas oil production system (Horacek and Gawel 1988). Finally, *Desulfobulbus* were enriched from seawater systems in UK oilfields (Vance and Brink 1994).

Desulfobacter was chosen as the candidate for development of a genus-specific DNA probe targeting ribosomal RNA because it had been reported from several other oil production systems and the natural environment. The purpose of this probe was to enable rapid detection and definitive identification of *Desulfobacter*.

Materials and methods

Culture

Desulfobacter enrichment medium (Vance and Brink 1994) was inoculated with produced water or injection seawater from an Alaskan oilfield. Eight samples were taken from the produced-water system. System temperatures were above 45°C for 6 of these samples and below 45°C for 2 samples. Fifteen samples were taken from the seawater system (all below 45°C); 5 of these were solids scrapings from bioprobes (Caproco Ltd., Edmonton, Alberta, Canada) or corrosion coupons which had been exposed to system conditions for 3 months. Samples were cultured at 30°C if they were from a part of the system operating at about 45°C or less, and duplicate enrichments were incubated at both 30°C and 55°C for samples taken from hotter parts of the system. Cultures exhibiting extensive bacterial growth were subcultured successively. *Desulfobacter* DB1 was isolated as a single colony in deep agar three successive times. The DB2 enrichment contained *Desulfobacter* at high population densities, and at least one other unidentified bacterial taxon at relatively low densities. *Desulfobacter* from oilfield systems were compared to a laboratory culture of *Desulfobacter* (3ac10), which was originally isolated from anaerobic mud flats of the North Sea (Widdel and Pfennig 1981). *Desulfobacter* were serially diluted into oilfield SRB medium (Cash et al. 1992) to determine whether they would be detected and enumerated by standard oilfield techniques.

DNA probes

Enrichment and isolation cultures were checked for the presence of *Desulfobacter* by using three different oligonucleotide DNA probes targeting ribosomal RNA, in a homogeneous, chemiluminescent, hybridization assay (Hogan 1991). The "eubacterial probe" targets a sequence of rRNA that is shared in common among eubacteria. The *Desulfovibrio Desulfotomaculum* probe targets several oilfield isolates of *Desulfovibrio* and *Desulfotomaculum*. The *Desulfobacter* probe targets a *Desulfobacter*-specific sequence of ribosomal RNA, which was shared in common among the six *Desulfobacter* analyzed by Devereux et al. (1989).

Hydrogenase assay

The hydrogenase assay measures the level of activity of hydrogenase, an enzyme that has been implicated in the cathodic depolarization mechanism of microbially influenced corrosion. This assay is marketed for microbiological monitoring of oilfield systems by Caproco Ltd., Edmonton, Alberta, Canada. The assay was performed following instructions provided by the manufacturer.

Rapidchek assay (adenylylsulfate reductase)

Rapidchek (marketed by Conoco Specialty Products, Houston, Texas), is an immunological assay for detection of SRB. It detects the presence of adenylylsulfate (APS) reductase, a metabolic enzyme of dissimilatory sulfate reduction, which should be ubiquitous among sulfate-reducing bacteria. The procedure for performing Rapidchek was modified to include an additional 3 ml distilled water wash as suggested for use with culture medium by the developers of the assay (R. Tatnall, personal communication).

Fatty acid analyses

Methods of fatty acid analysis have been described elsewhere (Dowling et al. 1986).

Desulfobacter detection

Detection and enumeration of *Desulfobacter* were compared using direct counting, serial dilution, DNA probes, and the hydrogenase

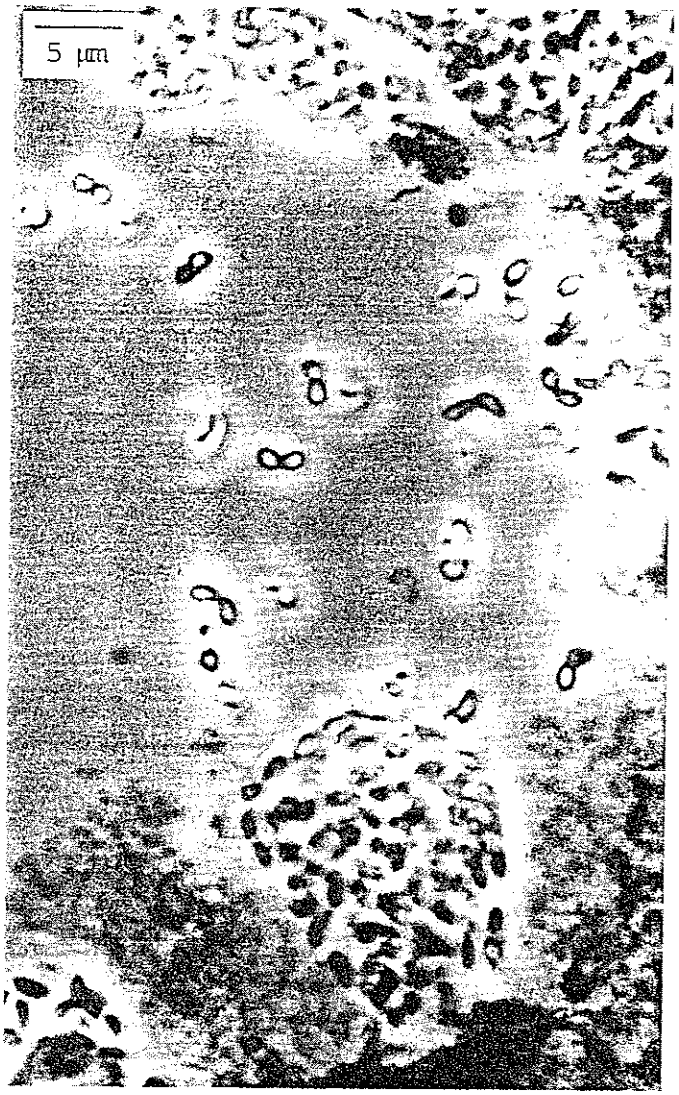

Fig. 1 *Desulfobacter* DB2 from seawater-injection wellhead. Paired cells, which are characteristic of *Desulfobacter*, were dislodged from the biofilm, which is evident at the *top*, *bottom* and *right edges* of the picture

and Rapidchek assays. Two serial-dilution media were tested, i.e., *Desulfobacter* medium (Widdel and Pfennig 1981), and the acetate/lactate- based enumeration medium of Cash et al. (1992), which has been used successfully for routine oilfield enumeration of SRB (primarily *Desulfovibrio* and *Desulfotomaculum*).

Results

Culture

Desulfobacter were recovered from 2 of 23 samples from various locations in Alaskan oil/water-handling systems. These *Desulfobacter* (DB1 and 2) were enriched from water samples taken from seawater-injection wellheads. They exhibited typical *Desulfobacter* morphology (Fig. 1). *Desulfobacter* DB1 and DB2 were similar,

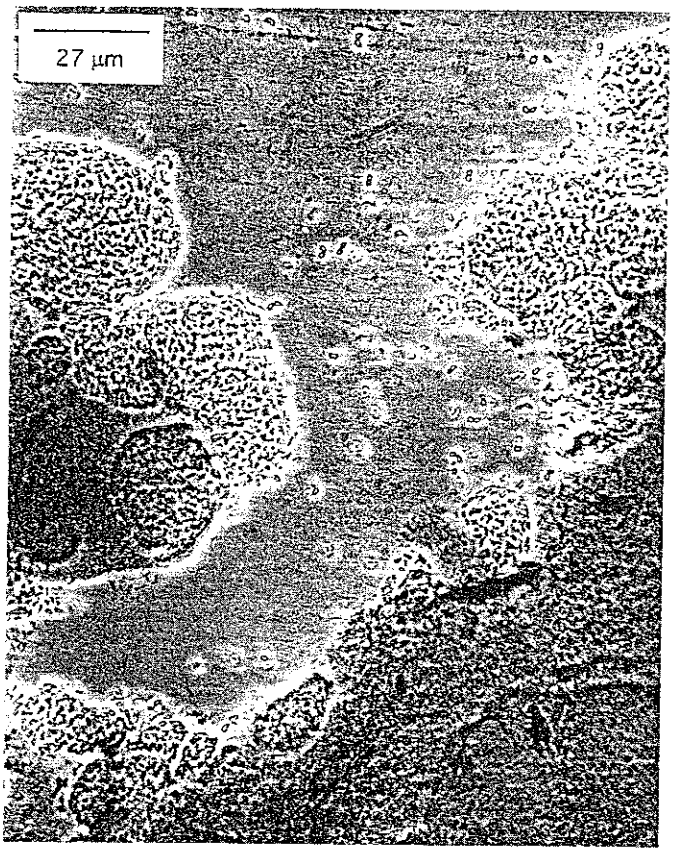

Fig. 2 *Desulfobacter* DB2, biofilm. Refractile (bright) areas are regions of living bacteria. Darker areas are older regions in the film

non-motile, hyaline, ellipsoidal to slightly fat, vibrioid with rounded ends, occurring singly or in pairs, 0.8–1.6 µm in diameter and 1.4–2.5 µm in length. Cells exhibiting the beginnings of division constriction reached 3.7 µm in length. Paired ellipsoidal cells were at an angle to each other, presenting a vibrioid appearance. In this regard, these oilfield *Desulfobacter* differ from 3ac10 cell pairs, which form straight rather than angled pairs. They were also unlike laboratory-strain 3ac10 in that the medium did not become turbid. These oilfield *Desulfobacter* produced brown flocs at the bottom of the culture vials which were composed of *Desulfobacter* cells in what appeared to be a matrix of extracellular polymer. Individual cells were difficult to disperse into the liquid medium, even with vigorous shaking. Microscopic examination of these flocs revealed a biofilm-like structure (Costerton et al. 1987), with highly refractile microcolonies of viable cells at the edges, and non-refractile areas containing dead cells (Fig. 2). Biofilm production diminished for *Desulfobacter* DB1 and DB2 that were maintained for several months by repeated subculture, and these cultures eventually became predominately planktonic in habit. Isolated colonies in deep agar were light to dark brown in color, with a lenticular colony shape. Some colonies were grown to almost 3 mm in diameter.

Table 1 *Desulfobacter* detection/enumeration comparing visual counts, serial dilution (sulfate-reducing bacteria, and *Desulfobacter*-specific medium), hydrogenase assay, Rapidchek, and DNA probes (targeting ribosomal RNA). The control was sterile *Desulfobacter* enrichment medium. Visual counts were made with a Petroff-Hausser chamber. Serial-dilution results are as bacteria/ml. Ribosomal-probe results are reported as relative light units (*RLU*) from 2 ml culture, and also as bacteria/ml from an empirically derived conversion factor, which is essentially (RLU − background) (5 bacteria/RLU) (ml^{-1}). All three cultures were manipulated for log-phase growth under the same conditions of transfer and incubation. Data are averages for replicates, which were in close agreement. *ND* not detected; *BDL* below detectable limits; *N/R* not reported because DB2 was a mixed culture, and these assays do not distinguish *Desulfobacter* from other bacteria

Desulfobacter	3ac10	DB1	DB2	control
Visual count (ml^{-1})	2.4×10^7	1.1×10^7	6.3×10^6	0
Serial dilution				
SRB medium	0	0	0	0
Desulfobacter medium	5×10^4	1×10^7	5×10^7	0
DNA probes				
Eubacterial probe				
Bacterial/ml	2.7×10^6	2.5×10^6	2.4×10^6	
RLU	1 082 900	1 018 500	946 750	4100
Desulfovibrio/Desulfotomaculum probe				
Bacteria/ml	BDL	BDL	BDL	
RLU	1100	950	850	1200
Desulfobacter probe				
Bacteria/ml	2.6×10^6	2.6×10^6	2.6×10^6	
RLU	1 045 800	1 028 450	1 038 850	3650
Hydrogenase assay	ND	Strong	N/R	ND
Rapidchek	ND	ND	N/R	ND

Standard SRB serial dilution medium (acetate/lactate), used for routine oilfield SRB enumeration, did not support growth of *Desulfobacter* 3ac10, DB1 or DB2. After bacteria had been adapted to laboratory culture and become predominately planktonic, serial dilution into *Desulfobacter* medium enumerated all three *Desulfobacter*, as expected (Table 1); it took as long as 18 days for the last bottle to become visually turbid.

DNA probes

DNA probes detected *Desulfobacter* with high sensitivity. This enabled rapid and definitive verification of the presence of *Desulfobacter* in the two acetate-based enrichment cultures with high densities of bacteria exhibiting the distinctive *Desulfobacter/Desulfobulbus* morphology. The presence of *Desulfobacter* was indicated by a relatively high signal with both eubacterial and *Desulfobacter* probes, compared to a lower (background) signal with the *Desulfovibrio/Desulfotomaculum* probe (Table 1). Signal strength varied with differences in population densities and metabolic activity (age), of the cultures (data not shown).

Some acetate-based mesophilic and thermophilic enrichments supported growth of diverse bacteria that lacked the distinctive *Desulfobacter* morphology. These were not detected by the *Desulfobacter* probe, and they were not subcultured.

Hydrogenase assay

Desulfobacter DB1 was strongly hydrogenase-positive (more than 5 nmol H_2 uptake/min). Hydrogenase activity was undetectable for *Desulfobacter* 3ac10 (Table 1).

Table 2 Phospholipid-ester-linked fatty acid (PLFA) profile for *Desulfobacter* isolate DB1. Fatty acids are distinguished by chain length and position of substituents from the methyl (ω) end. Thus, 16:1ω7*c* is ω7-*cis*-hexadecanoic acid. *i* (iso) and *a* (anteiso) indicate methyl-branching, one and two carbons from the methyl end respectively. Unsaturation may be *cis* (*c*) or *trans* (*t*). *br* multiple branched, *cy* cyclopropyl ring (see Dowling et al. 1986).

Phospholipid fatty acid	Composition (%)	Phospholipid fatty acid	Composition (%)
*i*14:0	0.3	16:1ω5c	2.2
14:1ω5*c*, *t*	1.3	16:0	37.2
14:0	11.3	*i*17:1(ω7*c*)[a,b]	0.4
*i*15:1(ω7*c*)[a]	0.6	*i*17:0	0.6
i15:0	5.9	a17:0/1ω8	0.2
*a*15:0	0.3	cy17:0[c]	9.4
cy15:0	0.1	17:0	0.2
15:0	0.6	18:1ω9*c*	0.3
*i*16:0	0.2	18:1ω7*c*	4.4
16:1(ω9*c*)[a]	1.5	18:0	0.6
16:1(ω7*c*)	22.1	br19:1	0.1
16:1(ω7*t*)	0.2	Other	0.3

[a] Requires further work for structure elucidation
[b] Biomarker for *Desulfovibrio desulfuricans* (Taylor and Parkes 1983). Obviously not dominant in the profile for isolate DB1
[c] Common PFLA for *Desulfobacter*

Rapidchek assay

Levels of adenylylsulfate reductase, as measured by the Rapidchek assay, were below detectable limits for *Desulfobacter* 3ac10 and DB1 (Table 1).

Fatty acid analyses

The phospholipid fatty acid profile for *Desulfobacter* DB1 is shown in Table 2.

Discussion

Culture

The most distinctive aspect of the two oilfield *Desulfobacter* was their extensive production of what was presumably an exopolysaccharide glycocalyx. Cell division produced sister cells that were trapped within the glycocalyx matrix, producing microcolonies. This has not been observed with our isolates of *Desulfovibrio* and *Desulfotomaculum*, which are planktonic rather than biofilm-ensconced in laboratory culture. Before the bacteria became predominately planktonic with adaptation to laboratory culture, it was necessary to disrupt the biofilm mechanically in order to photograph numerous *Desulfobacter* cells that were free of the film (Figs. 1, 2). It is thought that bacteria in biofilms are more damaging to oilfield systems than planktonic bacteria, because corrosion occurs at the metal/biofilm interface as a result of electrical and chemical heterogeneity within the film (Costerton et al. 1987).

Failure of *Desulfobacter* to grow in standard oilfield serial-dilution medium suggests that in most situations where these bacteria do occur, they are probably not being detected.

DNA probes

On the basis of ribosomal RNA sequence comparisons, the *Desulfobacter* probe would not be expected to detect other bacteria under the hybridization conditions used. Even the closest relatives of *Desulfobacter*, such as *Desulfobacterium*, differ by at least several nucleotides in the probe region (Devereux et al. 1989; rRNA sequence database). In practice, the probe successfully distinguished 18 *Desulfobacter* from other genera of SRB (Hogan 1991). Thus, if an isolate yields a strong signal when the *Desulfobacter*-specific probe is used, this is good evidence that the isolate is indeed *Desulfobacter*, or perhaps a very close relative. However, negative results with the probe would not necessarily prove that an isolate was not *Desulfobacter*. The probe sequence was based upon homology in the probe region among the six *Desulfobacter* 16S rRNA sequences that were available. This does not guarantee probe homology in future *Desulfobacter* isolates.

Hydrogenase assay

Some *Desulfobacter* exhibit high levels of hydrogenase activity (e.g. DB1, *D. hydrogenophilus*), while other strains lack hydrogenase activity (e.g., 3ac10, see also Pankhania 1988). Hydrogenases may participate in corrosion mechanisms long after the cells that produced the enzymes have died (Chatelus et al. 1987; Bryant and Laishley 1990). This suggests that hydrogenases might accumulate even in dead films of DB1 and continue to contribute to microbiological corrosion mechanisms.

Rapidchek

The Rapidchek assay (Dupont/Conoco) did not detect *Desulfobacter* isolates 3ac10 and DB1 even though about 10^8 active cells were used in the assay (Table 1). Adenylylsulfate reductase is a metabolic enzyme of dissimilatory sulfate reduction, and occurrence of the enzyme should be ubiquitous among SRB. Although this assay cannot be used to distinguish or identify particular genera of SRB, theoretically the assay should detect many kinds of SRB, assuming that the reductases from diverse genera are sufficiently similar to be cross-reactive with the antibodies used in the assay. However, there are differences among adenylylsulfate reductase from various SRB (Skyring and Trudinger 1973; Odom et al. 1991). It is possible that Rapidchek antibodies had low sensitivity for adenylylsulfate reductase from *Desulfobacter* 3ac10 and DB1. We understand that a second version of the Rapidchek assay has been modified to detect *Desulfobacter*.

Fatty acid analyses

The phospholipid fatty acid profile for oilfield isolate *Desulfobacter* DB1 is similar to that of *Desulfobacter latus* and the unnamed "fat vibrio" (Dowling et al. 1986; Widdel 1987). The lack of 10Me16:0 fatty acid in *Desulfobacter* DB1 was surprising because that fatty acid has been used as a "biomarker" for *Desulfobacter* (Horacek and Gawel 1988; Dowling et al. 1986; Parkes 1987). Caution, however, has been recommended in the use of this biomarker following studies of acetate-amended marine sediments in which the 10Me16:0 content was not enriched, despite demonstrable sulfate reduction at the expense of acetate (Parkes et al. 1993). Oligonucleotide probes targeting ribosomal RNA are a realistic alternative to phospholipid fatty acid profiles for identification of *Desulfobacter*.

Desulfobacter in the oilfield environment

Acetate is an abundant carbon source and electron donor available to microorganisms in oilfield-produced waters (Carothers and Kharaka 1978). The oilfield-produced waters used in this study for *Desulfobacter* enrichment have acetate concentrations in the approximate range 118–1090 mg/l, but the water system is too hot (above 55°C), to permit growth of *Desulfobacter* (Widdel 1987; Widdel and Pfennig 1982, 1984).

Acetate is below 1 mg/l in the seawater from which *Desulfobacter* were isolated. However, lack of acetate in the water phase would not necessarily be expected to preclude growth of *Desulfobacter*. Although *Desulfobacter* are selectively enriched using acetate, other carbon sources can be utilized including ethanol, pyruvate, propanol, butanol, higher fatty acids and benzoate (Widdel 1987; Widdel and Pfenning 1984). The *Desulfobacter* recovered from oilfield systems in this study had a sessile habit, and natural biofilms harbor diverse bacteria. This produces a "digestive consortium" (Costerton et al. 1987), in which nutrients produced by one kind of bacterium, or released by cell death, can be utilized by other kinds of bacteria. Thus, nutrients are trapped and recycled in the film, and bacteria are not limited to nutrients in the bulk water phase.

Conclusions

Desulfobacter are present in oilfield seawater systems, but in this study they were neither ubiquitous nor at high population levels. The *Desulfobacter*-specific DNA probe had practical utility in oilfield microbiology, especially when used in conjunction with enrichment culture. DNA probes have been used for several years for routine bacterial enumeration in Alaskan oilfield systems. Field use of the *Desulfobacter* probe, however, was discontinued because results were consistently below the detection limits of the hybridization assay (approx. 10^4 bacteria/ml), even for oilfield samples that yielded high numbers (10^6 + bacteria/ml), with the eubacterial probe.

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