Microbial Ecology

Molecular Assessment of Inoculated and Indigenous Bacteria in Biofilms from a Pilot-Scale Perchlorate-Reducing Bioreactor

H. Zhang[1], B.E. Logan[1], J.M. Regan[1], L.A. Achenbach[2] and M.A. Bruns[3]

(1) Department of Civil and Environmental Engineering, Pennsylvania State University, University Park, PA 16802, USA
(2) Department of Microbiology, Southern Illinois University, Carbondale, IL 62901, USA
(3) Department of Crop and Soil Science, Pennsylvania State University, University Park, PA 16802, USA

Received: 16 December 2003 / Accepted: 10 April 2004 / Online publication: 7 July 2005

Abstract

Bioremediation of perchlorate-contaminated groundwater can occur via bacterial reduction of perchlorate to chloride. Although perchlorate reduction has been demonstrated in bacterial pure cultures, little is known about the efficacy of using perchlorate-reducing bacteria as inoculants for bioremediation in the field. A pilot-scale, fixed-bed bioreactor containing plastic support medium was used to treat perchlorate-contaminated groundwater at a site in Southern California. The bioreactor was inoculated with a field-grown suspension of the perchlorate-respiring bacterium *Dechlorosoma* sp. strain KJ and fed groundwater containing indigenous bacteria and a carbon source amendment. Because the reactor was flushed weekly to remove accumulated biomass, only bacteria capable of growing in biofilms in the reactor were expected to survive. After 26 days of operation, perchlorate was not detected in bioreactor effluent. Perchlorate remained undetected by ion chromatography (detection limit 4 μg L^{-1}) during 6 months of operation, after which the reactor was drained. Plastic medium was subsampled from top, middle, and bottom locations of the reactor for shipment on blue ice and storage at −80°C prior to analysis. Microbial community DNA was extracted from successive washes of thawed biofilm material for PCR-based community profiling by 16S-23S ribosomal intergenic spacer analysis (RISA). No DNA sequences characteristic of strain KJ were recovered from any RISA bands. The most intense bands yielded DNA sequences with high similarities to *Dechloromonas* spp., a closely related but different genus of perchlorate-respiring bacteria. Additional sequences from RISA profiles indicated presence of representatives of the low G+C gram-positive bacteria and the *Cytophaga–Flavobacterium–Bacteroides* group. Confocal scanning laser microscopy and fluorescence *in situ* hybridization (FISH) were also used to examine biofilms using genus-specific 16S ribosomal RNA probes. FISH was more sensitive than RISA profiling in detecting possible survivors from the initial inoculum. FISH revealed that bacteria hybridizing to *Dechlorosoma* probes constituted <1% of all cells in the biofilms examined, except in the deepest portions where they represented 3–5%. Numbers of bacteria hybridizing to *Dechloromonas* probes decreased as biofilm depth increased, and they were most abundant at the biofilm surface (23% of all cells). These spatial distribution differences suggested persistence of low numbers of the inoculated strain *Dechlorosoma* sp. KJ in parts of the biofilm nearest to the plastic medium, concomitant with active colonization or growth by indigenous *Dechloromonas* spp. in the biofilm exterior. This study demonstrated the feasibility of *post hoc* analysis of frozen biofilms following completion of field remediation studies.

Introduction

For decades, discharges of perchlorate-containing wastes were not regulated during the manufacture and disposal of large quantities of ammonium perchlorate, primarily used as oxidizers in rocket propellants and munitions [17, 27]. As many as 75 perchlorate release sites in 22 states have been identified in the United States as potential sources of perchlorate contamination of ground and surface waters [36]. The only known natural sources of concentrated perchlorate salts are Chilean nitrate deposits, which, when used in agricultural fertilizers, might contribute to some low-level perchlorate dissemination [39]. Other recently identified sources of perchlorate include natural evaporites and electrochemical

Correspondence to: M.A. Bruns; E-mail: mvb10@psu.edu

DOI: 10.1007/s00248-004-0273-6 • Volume 49, 388–398 (2005)

reactions occurring in storage tanks and pipelines. The latter reactions were implicated when high perchlorate concentrations were detected in a water storage tank in Levelland, Texas [13]. It is now recognized that perchlorate contamination of subsurface waters at low parts-per-billion concentrations may be much more widespread than previously thought.

Perchlorate is highly soluble, stable, and mobile in aqueous systems [17]. Recent studies have raised concerns that perchlorate, although used as a drug to treat thyrotoxicosis [45], might also inhibit the production of thyroid hormones in healthy populations. Based upon recent epidemiological and toxicological studies, the U.S. Environmental Protection Agency's January 2002 draft report recommended a reference dose of 0.00003 mg kg^{-1} day^{-1}. This dose would result in a drinking water standard of 1 μg L^{-1}, using the assumed factors of 70 kg body weight and 2 L of water consumption per day [36]. The California Department of Health Services has responded to the draft report by reducing their Action Level for perchlorate from 18 μg L^{-1} to 4 μg L^{-1}, a level very close to the reported perchlorate detection limit (2.5 to 4 μg L^{-1}) using chromatography-based methods.

Abiotic reduction of perchlorate can be very slow as a result of the high activation energy required for this process [19]. However, bacteria capable of reducing perchlorate during anaerobic respiration are widespread in nature [15, 35, 46], and perchlorate toxicity in bacteria has been observed only at very high concentrations of 5 g L^{-1} [39]. Perchlorate-reducing bacteria have been isolated from numerous habitats presumably never exposed to perchlorate (although more comprehensive testing may lead to a reassessment of extant environmental perchlorate concentrations). A variety of laboratory-isolated pure cultures can use diverse electron donors for complete reduction of perchlorate to chloride via $ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$ [37]. The first two reactions are catalyzed by dissimilatory perchlorate and chlorate reductases [28], whereas the third reaction is catalyzed by the nonrespiratory enzyme chlorite dismutase, induced during perchlorate reduction [12, 15]. Oxygen produced by the third reaction is apparently rapidly consumed, because oxygen concentrations as low as 2 mg L^{-1} can inhibit perchlorate reduction [12, 40]. Perchlorate-reducing bacteria are either facultative anaerobes or microaerophiles that use oxygen preferentially as electron acceptor [8, 14, 15, 20, 28, 37, 43, 46, 48]. Many but not all perchlorate-reducing bacteria are also capable of dissimilatory nitrate reduction, suggesting that the two pathways are separate [8, 15].

The β-subdivision of Proteobacteria contains two groups of closely related perchlorate degraders, *Dechloromonas* spp. and *Dechlorosoma* spp., which are particularly widespread in the environment [1]. The most thoroughly characterized representatives of these two groups are *Dechloromonas agitata* and *Dechlorosoma suillum*, respectively. Although *D. suillum* is now recognized as *Azospira suillum* [41], we refer to the latter group of perchlorate degraders in this report as *Dechlorosoma* spp. Both groups include representatives that can reduce both perchlorate and nitrate [12]. One such strain, *Dechlorosoma* sp. KJ, was inoculated into a pilot-scale packed-bed bioreactor at a site in California for *ex situ* treatment of groundwater contaminated with both perchlorate and nitrate [31]. Although other engineered bioreactor systems have also been designed and used to treat perchlorate-contaminated water [7, 18, 34, 42], little is known about how well the bioaugmented microbes can compete with indigenous populations from the contaminated sites. Interestingly, in a study of trichloroethylene (TCE) degradation using microcosms inoculated with *Burkholderia* sp., it was concluded that TCE degradation was due not to the inoculated cultures, but to activity by indigenous populations selected by the operating conditions in the microcosms [33].

The objective of the current study was to analyze biofilm material from frozen samples of reactor packing to determine whether the inoculated strain, *Dechlorosoma* sp. KJ, could be detected after 6 months of bioreactor operation. To this end we extracted biofilm community DNA for RISA (ribosomal intergenic spacer analysis) [6] and examined biofilm material using fluorescent *in situ* hybridization (FISH) with oligonucleotide probes specific for *Dechlorosoma* and *Dechloromonas* spp. We hypothesized that the indigenous microorganisms from groundwater had colonized the bioreactor and outcompeted the inoculated strain.

Materials and Methods

Bioreactor Inoculum. *Dechlorosoma* sp. strain KJ was originally isolated from a bench-scale, perchlorate-reducing bioreactor [28]. This isolate was subsequently shown to reduce nitrate and chlorate as well as perchlorate, but not sulfate [24, 28]. Because KJ could also reduce nitrate, it was selected as the inoculum strain to treat groundwater containing perchlorate as well as nitrate. Prior to inoculation, KJ was grown for 1 week in a nitrogen-gas-purged 208-L drum containing groundwater amended with 300 mg L^{-1} $NaClO_4$, 450 mg L^{-1} $NaCH_3COO$, and a combined phosphate buffer–nitrogen solution of 1.92 g K_2HPO_4, 0.98 g $NaH_2PO_4H_2O$, and 0.5 g $NH_4H_2PO_4$ L^{-1} [31]. Perchlorate degradation was confirmed in the drum, and the cell suspension amended once more with perchlorate and nutrients before being pumped into the reactor, which was then left undisturbed for 1 day to allow bacteria to attach to media surfaces. The reactor was operated for 11 days in full recirculation

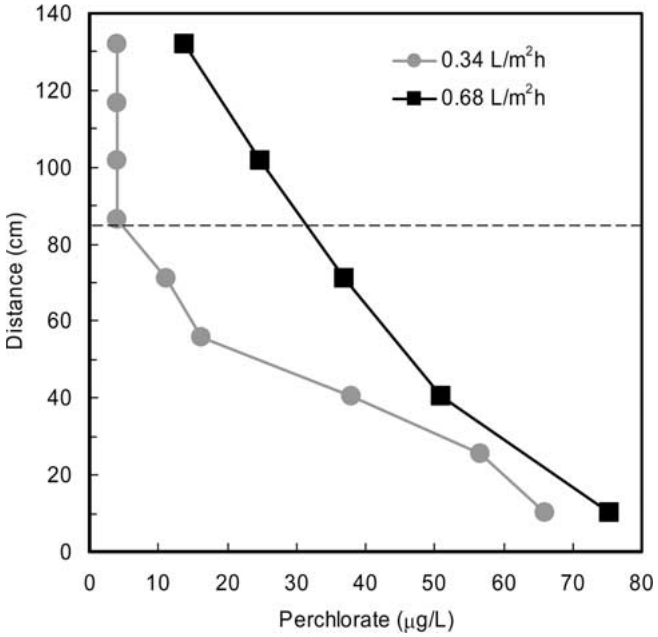

Figure 1. Perchlorate concentration profile in the reactor with two flow rates. Adapted from Min et al. [31].

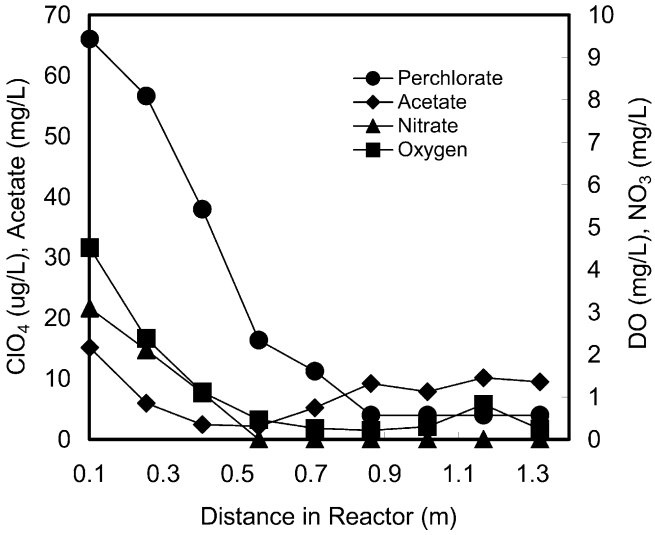

Figure 2. Chemical profiles in the reactor of acetate, oxygen, nitrate, and perchlorate at 0.34 L m^{-2} s^{-1} flow rate. Adapted from Min et al. [31], reprinted with permission.

mode using the 208-L drum and then switched to continuous-feed mode for groundwater treatment.

Bioreactor Operation and Sampling. Bioreactor dimensions were 2.1 m in height, 0.61 m in width, and 0.30 m in length. The bioreactor compartment was packed to a height of 1.2 m with plastic medium (Tripack, Jaeger Products) having 3.175-cm diameter and specific surface area of 230 m^2 m^{-3} [31]. A perforated plate was placed on top of the plastic medium bed to keep it in the reactor compartment because the density of the plastic was less than that of water. Groundwater stored in an equalization tank was pumped in upflow mode into the bottom of the bioreactor. At the top of the reactor, water overflowed a weir for transport to a drainage system by gravity [31]. Two different hydraulic loading rates of 0.34 and 0.68 L m^{-2} s^{-1} were tested. These rates corresponded to flows of 3.8 and 7.6 L min^{-1} and hydraulic detention times of 56 min and 28 min, respectively. Perchlorate was undetectable in bioreactor effluent at a flow rate of 0.34 L m^{-2} h^{-1}, but it was detectable at 0.68 L m^{-2} h^{-1}, as shown by the intracolumn perchlorate profiles in Fig. 1. After 26 days of operation at the lower rate, no perchlorate was detected in bioreactor effluent and it remained undetectable throughout the remainder of the test period. To remove excess biomass and prevent clogging or short circuiting, the reactor was also flushed from the bottom on a weekly basis by simultaneously increasing groundwater flow and aerating with a compressed air tank (2.6×10^{-3} m^3 s^{-1}) for 10 min.

The reactor was fed groundwater containing perchlorate (50–120 μg L^{-1}), dissolved oxygen (8–10 mg L^{-1}), nitrate (4–4.5 mg L^{-1} as N), and sulfate (33 mg L^{-1}), as well as added acetic acid (50 mg L^{-1}) and ammonium phosphate (12.8 mg L^{-1}). No effort was made in this study to optimize nutrient concentrations, and acetic acid and nitrogen–phosphate solutions (average C:N molar ratio of 5:1) were added in excess to avoid nutrient limitation. After 6 months of operation, flow was turned off and the water drained from the reactor compartment. Individual pieces of the plastic medium were collected aseptically from the top, middle, and bottom of the reactor. Samples were placed in separate Ziploc bags, shipped to Penn State overnight on blue ice, and stored at −80°C for 1 year until analysis.

Water Testing. Nine taps were located along one side wall of the compartment to permit water sampling at different heights. Perchlorate, acetate, nitrate, and sulfate were measured using ion chromatography (Dionex) as previously described [31]. Dissolved oxygen (DO) (minimum detection level of 0.3 mg L^{-1}) and oxidation–reduction potential (ORP) were measured using field instruments (YSI 600 XL). The average influent perchlorate concentration in the groundwater over 185 days of operation was 75 ± 13 μg L^{-1} (range 59–118 μg L^{-1}; $n = 94$). The bioreactor did not completely remove acetic acid, the concentration of which averaged 20 ± 8 mg L^{-1} at the outlet (Fig. 2). Intra-column profiles indicated sequential removal of dissolved oxygen, nitrate, and perchlorate (to less than 1 mg L^{-1}), but no sulfate removal was observed. Both oxygen and nitrate were undetectable after the water had reached a distance of

Table 1. FISH probe sequences, target sites, formamide concentrations in the hybridization buffer, and sodium chloride concentrations in the washing buffer

Probe	*Target 16S rRN positions (E. coli numbering)*	*Probe sequence*	*% formamide in hybridization buffer*	*NaCl (mM) in the wash buffer (mM)*	*References*
EUB338	338–355	5′-GCTGCCTCCCGTAGGAAGT−3′	20	225	[3]
NONEUB338	338–355	5′-ACTCCTACGGGAGGCAGC-3′	20	225	[43]
Monas1403	1403–1419	5′-GCGGAACCCGCTCCCAT-3′	20	225	This study
Soma1035	1017–1035	5′-CCATCTCTGGAAAGTTCCTGG-3′	0	900	This study

0.56 m from the inlet. Perchlorate was undetectable at 0.85 m. Influent and effluent DO means and standard deviations were 8.7 ± 0.4 and 0.2 ± 0.3 mg L^{-1}, respectively (Fig. 2). Influent and effluent ORP means and standard deviations were 8 ± 52 and −85 ± 77 mV, respectively.

DNA Extraction, PCR, Cloning, and 16S rRNA Gene Sequencing. To obtain biomass samples, three or four pieces of plastic medium were aseptically cut to fit into a 50-mL Falcon tube with 8 mL Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and vortexed at high speed for 1 min. The buffer was decanted and centrifuged to recover cell material from this ''first wash.'' The same plastic pieces were rinsed with fresh buffer and drained before another 8 mL buffer was added and the sample was vortexed and decanted a second time. This wash procedure was repeated a third time. Biomass samples were designated by their location in the bioreactor (top, T; middle, M; bottom, B) and by the number of washes prior to their recovery (1, 2, 3). Cell pellets were obtained from wash suspensions by centrifugation. Microbial community DNA was extracted from the cell pellets by a modified phenol–chloroform method [9]. The quantity and quality of DNA in extracts was evaluated by absorbances at 260 and 280 nm using a UV spectrophotometer as well as by inspection of stained bands after electrophoresis in 0.7% (w/v) agarose gels [38].

Bacterial community DNA (10 ng) was added as template in polymerase chain reactions (PCR) using a universal bacterial primer set which amplifies the 3′-ends of 16S rRNA genes, along with the 16S-23S intergenic spacer regions and short 5′-end pieces of 23S rRNA genes [10]. Because the lengths of bacterial spacers vary over a range of 80–1500 bp, the amplification products can be separated by size through gel electrophoresis to obtain bacterial community profiles [6]. Individual DNA bands can be excised from the gels and cloned to obtain sequences (∼600 bp) from 3′-portions of the 16S rRNA genes. We used forward and reverse primers 926F (5′-AAACTYAAAKGAATTGACGG-3′) and 23S115R (5′-GGGTTBCCCCATTCGG-3′), respectively [26]. The PCR products were separated by electrophoresis (80 V for 16 h) in 8% polyacrylamide gels, which were stained with SYBR Green (Molecular Probes, Eugene, OR) for visualization by UV illumination. Gel bands were excised with a razor blade, and DNA was eluted from the bands, extracted, and precipitated by using standard methods [38]. The DNA was ligated into a plasmid vector (TOPO TA, Invitrogen) for cloning into competent *E. coli* cells. DNA sequences were obtained from at least two purified transformants for each band (ABI Hitachi 3100). Sequence editing and alignment were conducted by using EditSeq and Seqman programs (Lasergene). The CHIMERA_CHECK [16] and Bellerophon [23] programs were used to evaluate sequences for chimeras. Closest relatives or the retrieved sequences were identified by searching GenBank with the Basic Local Alignment Search Tool [2].

Preparing Samples for in Situ Hybridization. Biofilm samples for hybridization and microscopic examination were obtained from thawed pieces of the plastic medium. A clean, ethanol-wiped scalpel was used to scrape off the surface layer of the plastic medium along with the biofilm in order to examine the entire depth of the biofilm on the plastic surface. The excised pieces (each approximately 6 mm by 6 mm) were placed on Multiwell coverslips (Molecular Probes) with the outer side of the biofilm facing the coverslip. In order to keep the biofilms from dislodging during the hybridization and washing steps, they were embedded in 10 μL 0.7% agarose [30]. Sample fixation, washing, and dehydration were performed following standard procedures [29].

Oligonucleotide Probes. The probe sequences, their complementary sites on 16S rRNA genes, and the conditions used in hybridization and washing are summarized in Table 1. The sequence of *Dechloromonas* probe Monas1403 was identified by a manual alignment with all other *Dechloromonas* and *Dechlorosoma* 16S rRNA sequences in GenBank. The *Dechlorosoma* probe Soma1035 was originally used as a PCR primer (data not shown). The specificity of the probes was evaluated by BLAST against the GenBank database, as well as by the CHECK_PROBE program from the Ribosomal Database Project II [16]. The 5′ end labels for probes EUB338, Monas1403, and Soma1035 were Cy5, Cy3, and FITC,

respectively. To confirm probe specificity, cell suspensions from pure cultures of *Dechloromonas* sp. HZ and *Dechlorosoma* sp. KJ were used as positive and negative controls. A nonsense probe, NONEUB338, whose nucleotide sequence was complementary to that of probe EUB338 was used as a control for nonspecific hybridization. Cell suspensions were prepared by fixation with paraformaldehyde at a final concentration of 1% for 90 min, washing with phosphate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate buffer at pH 7.2), resuspension in 1:1 mixture of PBS:96% ethanol, and storage at −20°C [4].

Fluorescent in Situ Hybridization. For cell suspensions, FISH was performed according to established procedures [3, 4]. Briefly, cells were spotted onto heavy Teflon-coated slides (Erie Scientific Company), air dried, dehydrated in an ethanol gradient, hybridized with oligonucleotide probes under various amounts of formamide, and washed with buffer containing appropriate amounts of sodium chloride (Table 1). For biofilm samples, hybridization was done at 46°C for 16 h in a moist chamber and washed at 48°C for 1 h as suggested by Moller et al. [32].

Image Acquisition and Quantitation of Biofilm Coverage on the Support Surface. Two separate biofilm samples from the top location were selected for finer-scale analysis by confocal laser scanning microscopy. To obtain images of cells stained with probes labeled with Cy3, Cy5, and FITC (fluorescein isothiocyanate), three excitation laser lines, red (633 nm), blue (488 nm), and green (543 nm), respectively, were used with an Olympus Fluoview 300 confocal microscope to acquire images in three independent channels. Images were viewed with a Plan-20 Apo 60× oil objective (numerical aperture 1.4). The *z* direction images were acquired at 3-μm intervals down into the biofilm. The 16-bit gray-scale images from each channel were first converted to the 8-bit gray-scale images required for COMSTAT input [21]. A fixed threshold value was used to generate binary images for each channel based on the ability to distinguish cells from background. The threshold gray values for Cy5, Cy3, and FITC channels were 80, 35, and 17, respectively. Images were examined manually at different depths throughout the *z* stacks, and no appreciable quenching of fluorescent signal was observed within the depth range used. Threshold-processed images were then subjected to COMSTAT analysis. Relative abundance of cells hybridizing with the Monas1403 and Soma1035 probes were determined by using the function of "area occupied by bacteria in each layer" in the COMSTAT program [21].

For the determination of biofilm thickness, agarose-embedded samples were stained with the *Bac*Light LIVE/DEAD viability dye (Molecular Probes). When stained, the nonliving biofilm matrix exhibited a uniform but low background fluorescence that was readily distinguishable from the brightly fluorescing, intact cells. The agarose exhibited very little fluorescence. The thickness of the biofilm was determined with an UplanFL 40× objective (numerical aperture = 0.75) by moving the focal plane away from the plastic surface until no fluorescence was observed. The distance between initial and final positions of the objective lens was used to calculate biofilm thickness. Estimates of mean biofilm thickness from top, middle, and bottom locations of the bioreactor were obtained from three or four independent measurements for each location. Total scanned areas for mean thickness calculations for top, middle, and bottom samples were 3.75×10^5, 3.74×10^5, and 4.99×10^5 μm^2, respectively.

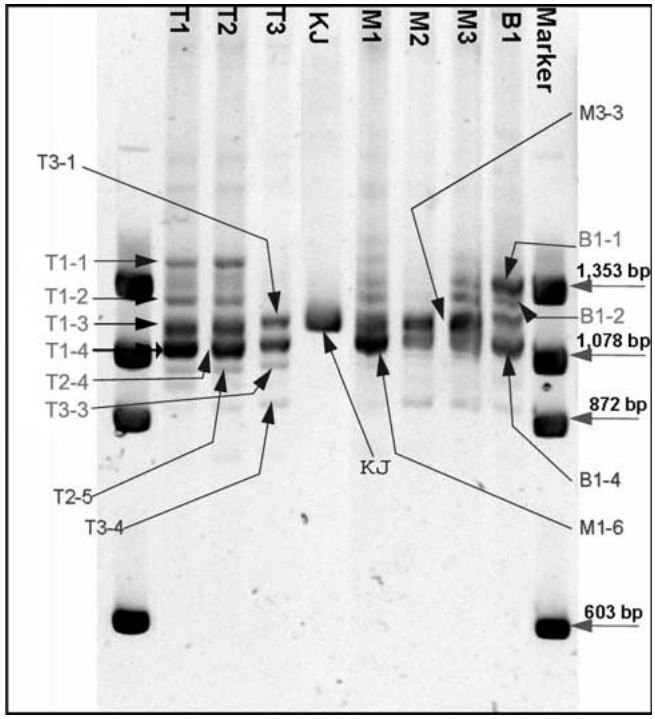

Figure 3. Inverted image of SYBR-Green stained polyacrylamide gel showing ribosomal intergenic spacer profiles of DNA extracted from the reactor biofilms.

GenBank accession numbers for partial 16S rRNA genes (*E. coli* positions 907–1542) are AY515710–AY515723.

Results

DNA was recovered from all three washes of plastic media from the top and middle locations, but only the first wash from the bottom location yielded enough DNA for PCR amplification. RISA community profiles from biofilm material removed by successive washes were compared to the RISA profile from genomic DNA of a

Table 2. Phylogenetic summary of perchlorate-reducing community from cloning and sequencing results (only 16S rDNA portion up to "GATCAC" were used in BLAST search)

Band and accession number	*Main group*	*Closest relatives in GenBank*	*Accession number*	*% similarity*
T1-1, B1-1 (AY510710, AY515714)	CFB	Clone S15A-MN90 (deep well groundwater in Russia)	AJ534684	98
T1-2, T3-1, M3-3 (AY515711, AY515720, AY515721)	Low G+C Gram pos	Clone:KU8 (coal tar waste–contaminated aquifer water)	AB074938	90
T1-4, B1-4 (AY515718, AY515722)	β-Proteobacteria	*Dechloromonas* sp. strain JJ	AY032611	98
T2-4 (AY515712)	β-Proteobacteria	*Dechloromonas* sp. strain HZ	AF479766	99
T2-5 (AY515719)	CFB	Clone IA-5 (chlorobenzene-treatment consortia)	AJ488076	94
T3-3, T3-4 (AY515713, AY515715)	Low G+C Gram pos	Clone ZZ12C4 (benzene-contaminated groundwater)	AY214180	98
M1-6 (AY515723)	β-Proteobacteria	*Dechloromonas* sp. strain JM	AF323489	100
B1-2 (AY515716)	CFB	*Flavobacterium psychrophilum*	AB078060	97

pure culture of *Dechlorosoma* sp. strain KJ (Fig. 3). None of the biofilm community profiles from the top location contained a band that migrated to the same position in the gel as the single RISA band from the *Dechlorosoma* sp. strain KJ culture. A putative KJ band could not be ruled out by visual inspection of the community profiles from the middle and bottom locations. Bands at positions nearest to that of KJ, however, yielded DNA sequences most closely related to representatives of the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group and low G+C gram-positive bacteria (Table 2). Although no sequences from *Dechlorosoma* sp. KJ were recovered from any RISA profile bands, biofilms from all bioreactor locations contained intense bands yielding DNA sequences from *Dechloromonas* spp., the other genus within the β-Proteobacteria which is known to reduce perchlorate [1]. Except for T1-3, which is a chimera detected by the Bellepheron program [23], all other sequences fell within the CFB and low G+C Gram-positive groups (Table 2).

Biofilms from the top and middle locations of the bioreactor were thicker than biofilms from the bottom location, which presumably reflected the effect of upward flushing from the reactor bottom. Measured thicknesses of biofilms from the top and middle locations were 96.7 ± 25.5 and 89.3 ± 12.9 μm (mean ± standard deviation, $n = 3$), respectively. The thickness of the biofilm from the bottom-location was 57.5 ± 22.5 μm ($n = 4$). Community profiles from the first and second washes of plastic packing from the top location did not contain the same bands as the community profile from the third wash, indicating qualitative differences in populations that could be dislodged with different degrees of washing force. The three bands having different positions in the T3 community profile all yielded DNA sequences belonging to low G+C Gram-positive bacteria (Table 2).

To assess presence and spatial distribution of *Dechlorosoma* and *Dechloromonas* spp. on a finer scale, we applied FISH probes Soma1035 and Monas1403, respectively, in conjunction with a universal EUB338 probe, to biofilm samples for analysis with confocal microscopy. Probe specificities were validated in hybridization tests with cells from pure cultures of the respective target organisms (Fig. 4A, B), and no cross hybridization was observed (data not shown). Nonspecific hybridization to probe NONEUB338 also was not observed. Confocal microscopic images of probe-hybridized biofilm samples are shown in Fig. 4C. Sequential analysis of z stack images indicated that most cells hybridizing to the Monas1403 probe (pink color in Fig. 4C) were in the exterior portions of the biofilm (0 and 15 μm depths). Similar distribution patterns were observed in three independently selected fields of view. Very low numbers of cells hybridized with the Soma1305 probe. These were indicated by signals from the green channel, which appeared whitish-blue in combination with the blue-channel signal from co-hybrization with EUB338 (arrows in last panel of Fig. 4C).

Relative abundances of bacteria hybridizing to the Monas and Soma probes were determined on the basis of total field-of-view areas using the COMSTAT program (Fig. 5A). Only 50% of total field area was covered by cells hybridizing to the EUB338 probe, with the remainder of the field area exhibiting a uniform background fluorescence. Cells hybridizing to the Monas and Soma probes covered 12% and <1% of total field areas, respectively. Relative abundances of *Dechloromonas* and *Dechlorosoma* spp. were also assessed in relation to total cells hybridizing with probe EUB338 (Fig. 5B). The results clearly showed that abundance of cells hybridizing with the Monas probe (*Dechloromonas* spp.) decreased with depth in the biofilm. While *Dechloromonas* spp. accounted for nearly 23% of bacterial biomass at the surface ($z = 0$ μm), this proportion decreased to only about 1% at a depth of 111 μm from the surface. Cells hybridizing with the Soma probe (*Dechlorosoma* spp.) represented <1% of bacterial biomass throughout most of the biofilm. At the bottom of the biofilm near the

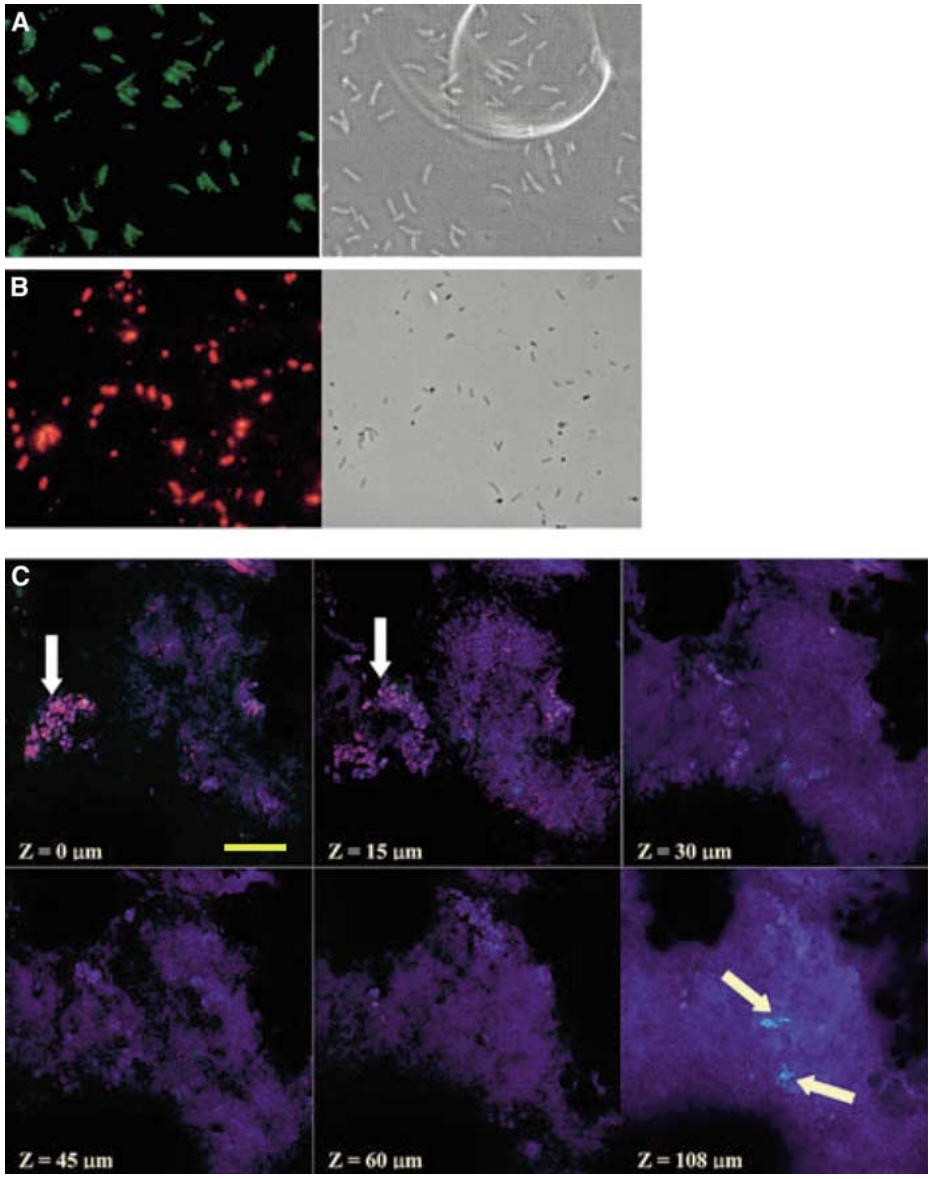

Figure 4. FISH detection of *Dechloromonas* and *Dechlorosoma* species. (A) FITC-labeled probe Soma1035 hybridization to the positive control *Dechlorosoma* sp. KJ. The same view showing the probe signal (green) and phase contrast image. (B) Cy3-labeled probe Monas1403 hybridization to the positive control *Dechloromonas* sp. HZ. The same view showing the probe signal (red) and phase contrast image. (C) Spatial distribution of *Dechloromonas* and *Dechlorosoma* species revealed by FISH-confocal imaging. Three separate color channels show signals from three probes: blue: Cy5 labeled EUB338, red: Cy3-labeled *Dechloromonas* probe Monas1403, green: FITC labeled *Dechlorosoma* probe Soma1035. Cells doubled-stained with EUB338 and Monas1403 appear pink (white arrows). Cells double-stained with EUB338 and Soma1035 appear whitish-blue (yellow arrows). The scale bar represents 20 μm.

plastic surface, however, *Dechlorosoma* spp. constituted ~5% of all bacterial biomass (Fig. 5B), suggesting that these may have been surviving populations from the original inoculum.

Discussion

After 6 months of operation following inoculation with *Dechlorosoma* sp. KJ, the perchlorate-reducing bioreactor supported biofilms comprising multiple species representing three phylogenetic groups: β-Proteobacteria, the CFB group, and low G+C Gram-positive bacteria. Biofilm populations derived from the *Dechlorosoma* sp. KJ inoculum were not in sufficiently high numbers to be detected by PCR-based community profiling. Instead, RISA detected greater abundances of members of the genus *Dechloromonas* spp., which are also known to reduce perchlorate. DNA sequences characteristic of *Dechloromonas* spp. were recovered from prominent bands in community profiles from all three locations of the bioreactor. Lack of detection by RISA of DNA sequences for *Dechlorosoma* spp. may have been due to the fact that KJ was inoculated only once into the bioreactor. Since the reactor was also flushed on a weekly basis to remove excess buildup of biomass, regular disturbance may have interfered with the ability of inoculated populations to persist [31]. Weekly flushing to remove excess biomass may also have favored the establishment of indigenous bacteria that were continually being reintroduced with the groundwater being treated.

Another reason why KJ did not become dominant in the biofilms could be the relatively low concentration of

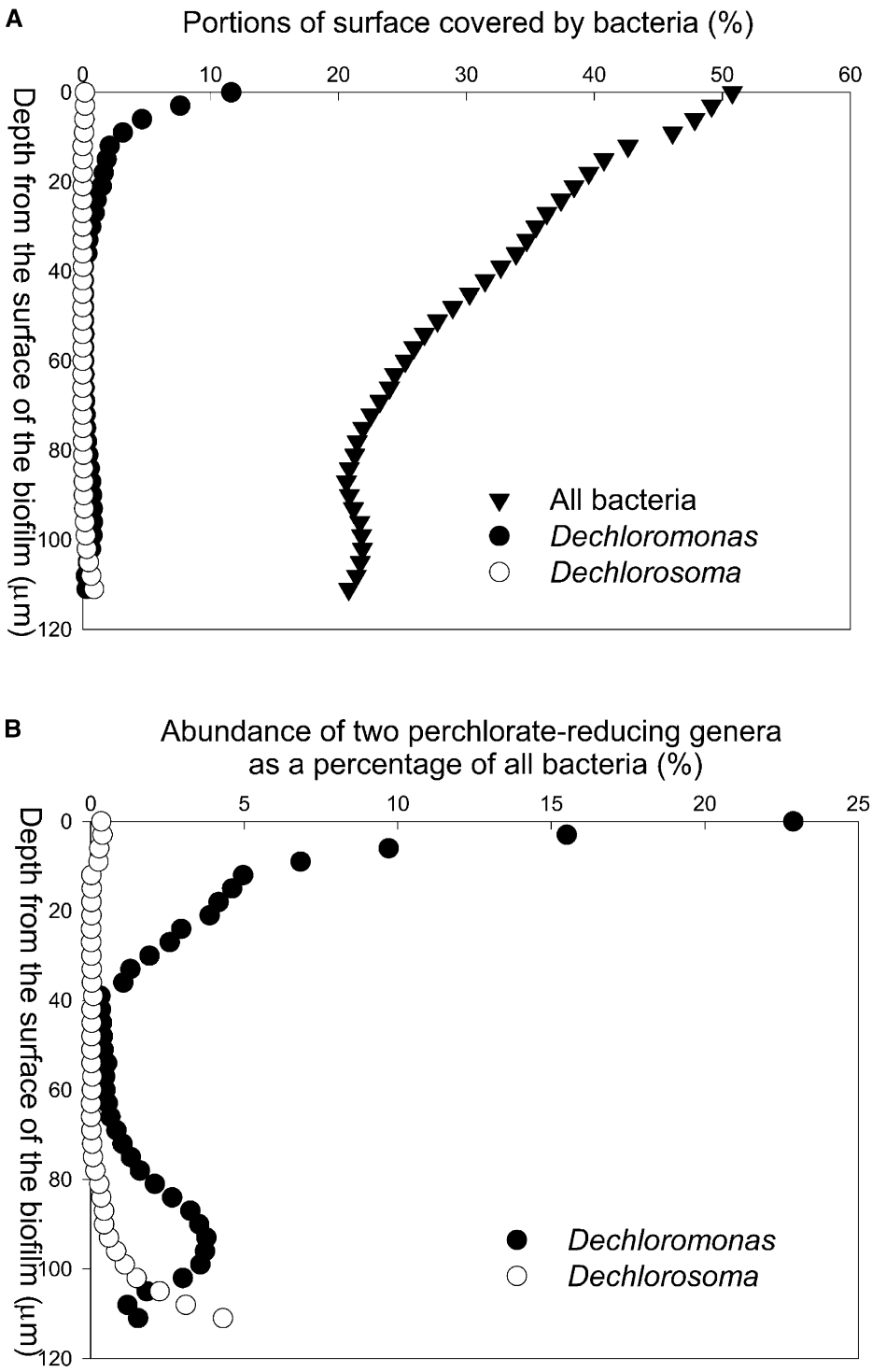

Figure 5. (A) Relative abundance of bacteria hybridizing with EUB338, Monas, and Soma probes on the basis of total field area. (B) Relative abundance of bacteria hybridizing with Monas and Soma probes on the basis of all bacteria. Data were obtained with the COMSTAT program.

perchlorate in the groundwater feed (0.05–0.12 mg L^{-1}), compared to the 300 mg L^{-1} perchlorate in the medium in which KJ had been grown and acclimated prior to inoculation of the bioreactor [31]. It should also be noted that KJ was originally isolated from a laboratory reactor fed with medium containing 20 mg L^{-1} perchlorate [24] and subsequently determined to have a half-saturation constant for perchlorate of 33 mg L^{-1} [28]. Indigenous bacteria in the groundwater could therefore have been adapted better to the bioreactor's perchlorate concentrations. Furthermore, perchlorate represented such a small proportion of the total electron acceptor pool that a very small population would have been sufficient to reduce it. Microbial populations, particularly those at the

bottom of the reactor, were exposed to influent concentrations of dissolved oxygen and nitrate that were 80-fold higher than that of perchlorate. The nutrient and operating conditions of the bioreactor would therefore not have enriched solely for perchlorate-reducing bacteria. There was also a significant amount of residual acetic acid in the reactor effluent (21 mg L^{-1}), indicating that electron donor availability was ample for diverse populations to become established.

In considering solely the perchlorate-reducing populations, the presence of dissolved oxygen and nitrate in the groundwater may also have selected for perchorate reducers that were less sensitive to fluxes in electron acceptors. In a study by Chaudhuri et al., *Dechlorosoma suillum* and *Dechloromonas agitata* strain CKB exhibited clear differences in activity when transferred from media containing only one type of electron acceptor (perchlorate or nitrate) to media containing both [12]. When transferred to media containing equimolar amounts of perchlorate and nitrate, nitrate-grown cells of *Ds. suillum* exhibited an extended lag, while perchlorate-grown cells preferentially reduced nitrate and did not reduce perchlorate until nitrate was removed completely. In contrast, *Dm. agitata* strain CKB reduced perchlorate equally well in the presence or absence of nitrate [12]. In other studies conducted on *Dechlorosoma* sp. KJ, chlorate and nitrate reduction pathways were observed to be induced separately, and the presence of as little as 5 μg NO_3^- L^{-1} inhibited its ability to reduce chlorate [47]. These studies are consistent with our observations in the nitrate-and-perchlorate-fed bioreactor, where cells hybridizing with the Monas1403 probe outnumbered cells hybridizing with the Soma1035 probe. The ability to simultaneously reduce nitrate and perchlorate would have been a selective advantage for populations in our bioreactor. Before this could be considered a definitive explanation for our results, however, other strains of *Dechloromonas* spp. would need to be evaluated for their ability to carry out simultaneous reduction of nitrate and perchlorate.

In our study, FISH probes could have hybridized to some β-proteobacteria belonging to genera other than *Dechlorosoma* and *Dechloromonas* spp. RDP II check_probe results for Monas1403 yielded a total of 17 perfect matches consisting of eight sequences from *Dechloromonas* spp., and nine sequences from other beta-Proteobacteria including two *Leptothrix*, one *Acidovorax*, and six unaligned sequences. Results for Soma1305 yielded seven matches, all within the *Rhodocyclus tennis* subgroup (four *Dechlorosoma* spp., two *Azoarcus*, and one uncultured clone). Evidence in our study which supports FISH detection of *Dechloromonas* populations, however, is the independent recovery of 600-bp rRNA gene sequences representative of *Dechloromonas* spp. from RISA profiles.

Based on the reactor chemical profiles in Fig. 2, DO and nitrate concentrations at the bottom location would not have enabled induction of perchlorate reduction by *Dechlorosoma* sp. KJ. Although the depletion of DO and nitrate at the middle and top locations would have been more permissive, rRNA gene sequences associated with the inoculum strain were not detected in RISA profiles at these locations. As expected, FISH and image analysis at a finer spatial scale were more sensitive in detecting less abundant populations. FISH showed that bacteria hybridizing to the *Dechlorosoma* probe were present in very low numbers in the deep interior of top-location biofilms (111 μm from the biofilm surface), although they were outnumbered by *Dechloromonas* spp. at more shallow depths (Fig. 5A, B). The numbers of cells hybridizing with the *Dechloromonas* probe increased with distance from the plastic medium, reaching their highest proportions of total bacterial biomass at the surface. These spatial differences suggested that *Dechloromonas* populations were introduced from groundwater.

Although the inoculated *Dechlorosoma* sp. strain KJ did not maintain a dominant presence in biofilms, the bioreactor was still effective at removing perchlorate, presumably because of the establishment and activity of perchlorate-reducing, indigenous populations. Our results are not the first to show that indigenous microorganisms can outcompete bioaugmented bacteria derived from laboratory strains. In one study of bioreactors treating mercury-contaminated wastes, two mercury-resistant inoculum strains disappeared after 485 days, whereas two different γ-Proteobacteria capable of reducing mercury invaded the biofilms [11]. In a microcosm study of TCE degradation, indigenous bacteria rather than inoculated strains were hypothesized to be responsible for TCE degradation [33].

Despite a long-held recognition that microorganisms cultured from environmental samples are often not the most dominant or active populations *in situ*, some laboratory enrichment and isolation procedures have nevertheless yielded closely related representatives of previously uncharacterized and important indigenous bacteria. In a controlled field study of *in situ* MTBE degradation in groundwater, for example, ribosomal RNA gene sequences with high similarity to laboratory strain PM1 were detected in higher numbers following a rise in MTBE concentration [22]. We present here another example of independent molecular detection of members of a recently recognized genus, *Dechloromonas*, which was identified through traditional cultural procedures and has demonstrated capabilities for degrading a specific pollutant [1, 15, 24, 28].

Representatives of the other phylogenetic groups detected in the biofilms are not known to have the ability to reduce perchlorate [1, 27]. Although their roles are unknown, members of the CFB group are widespread and known to utilize diverse carbon substrates [25]. The rRNA gene sequences belonging to the CFB group in our

bioreactor had high similarities to GenBank accessions from diverse environments, including deep well groundwater at a site in Russia (clones from T1-1 and B1-1), and stable bacterial consortia for chlorobenzene treatment (clone from T2-5). The five rRNA gene sequences recovered from our reactor that belonged to low G+C Gram-positive bacteria were genetically less diverse than the CFB sequences. Three of the five clones (from T1-2, T3-1, and M3-4) had identical 16S rRNA gene sequences (600 nt at 3′ ends) with greatest similarity (90%) to a GenBank accession from coal tar waste–contaminated aquifer waters [5]. The other two rRNA gene sequences (from T3-3 and T3-4) both had highest similarities (99%) to a GenBank accession from benzene-contaminated groundwater. The recovery of nearly identical rRNA gene sequences from bands in different locations of the RISA gels indicated that some biofilm populations, in particular Gram-positive bacteria, could have had more than one ribosomal RNA operon.

In this study we followed a stepwise molecular approach to analyzing biofilm communities in the perchlorate-degrading bioreactor. In the initial, exploratory step, we used RISA profiling with universal bacterial primers to identify dominant populations and discern qualitative differences based on bioreactor location and ease of removal from the plastic medium. RISA analysis enabled us to identify bacterial populations other than the inoculated strain which appeared to be dominant in biofilm communities. Second, cloning and sequencing of the 16S rRNA gene portions of excised RISA bands provided the basis for selecting the most informative probes for key populations. Third, we tried FISH analysis to obtain finer scale resolution and more quantitative estimates of biofilm populations, recognizing that freezing and thawing of the biofilm samples could have affected biofilm integrity. Although only a very small fraction of total surface area of plastic media was analyzed in our study, our objective was not to conduct a comprehensive spatial analysis of the bioreactor but to assess the feasibility of *post hoc* molecular techniques. Our study demonstrated that FISH can be performed successfully on some biofilm materials even after frozen storage and that FISH might be applicable in other studies to relate biofilm community composition to remediation performance. Finally, use of genus-specific probes in FISH analysis permitted quantification of population sizes in relation to biofilm depth and provided the basis for evaluating the utility of bioaugmentation in this pilot-scale system.

Acknowledgments

We thank Arne Heydorn for providing the COMSTAT program and Ian Head for review comments. This research was funded by the American Water Works Association Research Foundation 2557. H.Z. was supported by the Penn State Biogeochemical Research Initiative for Education (BRIE) sponsored by NSF (IGERT) grant DGE-9972759.

References

1. Achenbach, L, Michaelidou, U, Bruce, R, Fryman, J, Coates, J (2001) *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position. Int J Syst Evol Microbiol 51: 527–533
2. Altschul, SF, Gish, W, Miller, W, Myers, EW, Lipman, DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410
3. Amann, RI, Binder, BJ, Olson, RJ, Chisholm, SW, Devereux, R, Stahl, DA (1990) Combination of 16S ribosomal-RNA-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56: 1919–1925
4. Amann, RI, Krumholz, L, Stahl, DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172: 762–770
5. Bakermans, C, Hohnstock-Ashe, AM, Padmanabhan, S, Padmanabhan, P, Madsen, EL (2002) Geochemical and physiological evidence for mixed aerobic and anaerobic field biodegradation of coal tar waste by subsurface microbial communities. Microb Ecol 44: 107–117
6. Borneman, J, Triplett, EW (1997) Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl Environ Microbiol 63: 2647–2653
7. Brown, JC, Snoeyink, VL, Kirisits, MJ (2002) Abiotic and biotic perchlorate removal in an activated filter. J Am Water Works Assoc 94: 70–79
8. Bruce, RA, Achenbach, LA, Coates, JD (1999) Reduction of (per)chlorate by a novel organism isolated from paper mill waste. Environ Microbiol 1: 319–329
9. Bruns, MA, Buckley, DH (2001) Isolation and purification of microbial community nucleic acid from environmental samples. In: Hurst, C, et al. (Ed.) Manual of Environmental Microbiology, 2nd ed., ASM Press, Washington, pp 564–572
10. Bruns, MA, Hanson, JR, Mefford, J, Scow, KM (2001) Isolate PM1 populations are dominant and novel methyl tert-butyl ether-degrading bacterial in compost biofilter enrichments. Environ Microbiol 3: 220–5
11. Canstein, HF, Li, Y, Felske, A, Wagner-Dobler, I (2001) Long-term stability of mercury-reducing microbial biofilm communities analyzed by 16S-23S rDNA interspacer region polymorphism. Microb Ecol 42: 624–634
12. Chaudhuri, SK, O'Connor, SM, Gustavson, RL, Achenbach, LA, Coates, JD (2002) Environmental factors that control microbial perchlorate reduction. Appl Environ Microbiol 68: 4425–4430
13. Christen, K (2003) Perchlorate mystery surfaces in Texas. Environ Sci Technol 37: 376A–377A
14. Coates, JD, Chakraborty, R, Lack, JG, O'Connor, SM, Cole, KA, Bender, KS, Achenbach, LA (2001) Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. Nature 411: 1039–1043
15. Coates, JD, Michaelidou, U, Bruce, RA, O'Connor, SM, Crespi, JN, Achenbach, LA (1999) Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Appl Environ Microbiol 65: 5234–5241
16. Cole, JR, Chai, B, Marsh, TL, Farris, RJ, Wang, Q, Kulam, SA, Chandra, S, McGarrell, DM, Schmidt, TM, Garrity, GM, Tiedje,

JM (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucl Acids Res 31: 442–443
17. Espenson, JH (2000) The problem and perversity of perchlorate. In: Urbansky, ET (Ed.) Perchlorate in the Environment, Kluwer Academic/Plenum, New York, pp 1–8
18. Giblin, T, Herman, D, Deshusses, MA, Frankenberger, WT (2000) Removal of perchlorate in ground water with a flow-through bioreactor. J Environ Qual 29: 578–583
19. Gu, B, Dong, W, Brown, GM, Cole, DR (2003) Complete degradation of perchlorate in ferric chloride and hydrochloric acid under controlled temperature and pressure. Environ Sci Technol 37: 2291–2295
20. Herman, DC, Frankenberger, WT (1999) Bacterial reduction of perchlorate and nitrate in water. J Environ Qual 28: 1018–1024
21. Heydorn, A, Nielsen, AT, Hentzer, M, Sternberg, C, Givskov, M, Ersboll, BK, Molin, S (2000) Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146Pt 102395–2407
22. Hristova, K, Gebreyesus, B, Mackay, D, Scow, KM (2003) Naturally occurring bacteria similar to the methyl tert-butyl ether (MTBE)-degrading strain PM1 are present in MTBE-contaminated groundwater. Appl Environ Microbiol 69: 2616–2623
23. Hugenholtz, P, Huber, T (2003) Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Int J Syst Evol Microbiol 53: 289–293
24. Kim, K, Logan, BE (2001) Microbial reduction of perchlorate in pure and mixed culture packed-bed bioreactors. Wat Res 35: 3071–3076
25. Kirchman, DL (2002) The ecology of *Cytophaga–Flavobacteria* in aquatic environments. FEMS Microbiol Ecol 39: 91–100
26. Lane, DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt, E, Goodfellow, M (Eds.) Nucleic Acid Techniques in Bacterial Systematics, John Wiley & Sons, New York, pp 115–175
27. Logan, BE (2001) Assessing the outlook for perchlorate remediation. Environ Sci Technol 35: 482a–487a
28. Logan, BE, Zhang, H, Mulvaney, P, Milner, MG, Head, IM, Unz, RF (2001) Kinetics of perchlorate- and chlorate-respiring bacteria. Appl Environ Microbiol 67: 2499–2506
29. Manz, W (1999) *In situ* analysis of microbial biofilms by rRNA-targeted oligonucleotide probing. Methods Enzymol 310: 79–91
30. Massol-Deya, A, Whallon, J, Hickey, R, Tiedje, JM (1995) Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater. Appl Environ Microbiol 61: 769–777
31. Min, B, Evans, PJ, Chu, A, Logan, BE (2004) Perchlorate removal in sand and plastic media bioreactors. Wat Res 38: 47–60
32. Moller, S, Pedersen, AR, Poulsen, LK, Arvin, E, Molin, S (1996) Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative *in situ* hybridization and scanning confocal laser microscopy. Appl Environ Microbiol 62: 4632–4640
33. Munakata-Marr, J, Matheson, VG, Forney, LJ, Tiedje, JM, McCarty, PL (1997) Long-term biodegradation of trichloroethylene influenced by bioaugmentation and dissolved oxygen in aquifer microcosms. Environ Sci Technol 31: 786–791
34. Nerenberg, R, Rittmann, BE (2002) Perchlorate as a secondary substrate in a denitrifying hollow-fiber membrane biofilm reactor. Wat Sci Technol 2: 259–265
35. Nozawa-Inoue M, Leong J, Rolston D, Scow KM (2003) Abstracts of the American Society for Microbiology General Meeting, May 18th to 22nd, Washington, DC
36. Renner, R (2003) Environmental health, Academy to mediate debate over rocket-fuel contaminants. Science 299: 1829
37. Rikken, GB, Kroon, AGM, vanGinkel, CG (1996) Transformation of (per)chlorate into chloride by a newly isolated bacterium: reduction and dismutation. Appl Microbiol Biotechnol 45: 420–426
38. Sambrook, J, Fritsch, EF, Maniatis, T (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY
39. Schilt, AA (1979) Perchloric Acid and Perchlorates. G.F. Smith Chemical Co., Columbus, OH
40. Song, Y, Logan, BE (2004) Effect of O_2 exposure on perchlorate reduction by *Dechlorosoma* sp. KJ. Wat Res 38: 1626–1632
41. Tan, Z, Reinhold-Hurek, B (2003) *Dechlorosoma suillum* Achenbach et al. 2001 is a later subjective synonym of *Azospira oryzae* Reinhold-Hurek and Hurek 2000. Int J Sys Evol Microbiol 53: 1139–1142
42. Wallace, W, Beshear, S, Williams, D, Hospadar, S, Owens, M (1998) Perchlorate reduction by a mixed culture in an up-flow anaerobic fixed bed reactor. J Indust Microbiol Biotechnol 20: 126–131
43. Wallace, W, Ward, T, Breen, A, Attaway, H (1996) Identification of an anaerobic bacterium which reduces perchlorate and chlorate as *Wolinella succinogenes*. J Indust Microbiol 16: 68–72
44. Wallner, G, Amann, R, Beisker, W (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14: 136–143
45. Wolff, J (1998) Perchlorate and the thyroid gland. Pharmacol Rev 50: 89–106
46. Wu, J, Unz, RF, Logan, BE (2001) Persistence of perchlorate and the relative numbers of perchlorate- and chlorate-respiring microorganisms in natural waters, soils and wastewater. Bioremed J 5: 119–130
47. Xu, J, Trimble, JJ, Steinberg, L, Logan, BE (2004) Chlorate and nitrate reduction pathways are separately induced in the perchlorate-respiring bacterium *Dechlorosoma* sp. KJ and the chlorate-respiring bacterium *Pseudomonas* sp. PDA. Wat Res 38: 673–680
48. Zhang, H, Bruns, MA, Logan, BE (2002) Perchlorate reduction by a novel chemolithoautotrophic, hydrogen-oxidizing bacterium. Environ Microbiol 4: 570–576