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Community structure and function in a H_2-based membrane biofilm reactor capable of bioreduction of selenate and chromate

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Abstract Two different H_2-based, denitrifying membrane-biofilm reactors (MBfRs) initially reduced Se(VI) or Cr (VI) stably to Se^0 or Cr(III). When the oxidized contaminants in the influent were switched, each new oxidized contaminant was reduced immediately, and its reduction soon was approximately the same or greater than it had been in its original MBfR. The precipitation of reduced selenium and chromium in the biofilm was verified by scanning electron microscopy and energy dispersive X-ray analysis. These results on selenate and chromate reduction are consistent with the interpretation that the H_2-based biofilm community had a high level of functional diversity. The communities' structures were assessed by cloning analysis. *Dechloromonas* spp., a known perchlorate-reducing bacteria, dominated the clones from both reactors during selenate and chromate reductions, which suggests that it may have functional diversity capable of reducing selenate and chromate as secondary and dissimilatory acceptors.

Introduction

Recently, several oxidized contaminants have emerged as drinking-water pollutants, including selenate $\left(SeO_4^{2-}\right)$ and chromate $\left(CrO_4^{-}\right)$. A variety of natural, agricultural, and industrial processes can cause selenium (Se) contamination of surface waters (Cantafio et al. 1996; Haygarth 1994; Oremland et al. 1994). High concentrations of soluble selenate [Se(VI)] and selenite [Se(IV)] are acutely and chronically toxic to organisms inhabiting aquatic environments (Gillespie and Baumann 1986; Haygarth 1994). Bioaccumulation of Se at Kesterson reservoir in California created a reproductive hazard to waterfowl in the early 1980s (Ohlendorf et al. 1986; Presser and Ohlendorf 1987). Chromium is an important metal commonly used is tanning, electroplating, and steel manufacture and it is a common industrial pollutant (Gadd and White 1993; Ajmal et al. 1996; Achterberg et al. 1999; Ernst and Nelissen 2000). Chromium in its hexavalent oxidation state $\left(CrO_4^{2-}\right)$ is a likely carcinogen and mutagen (Langard 1982).

Conventional water-treatment techniques are not effective in removing these compounds. Advanced treatment techniques, such as reverse osmosis, ion exchange, membrane filtration, and electodialysis, are effective, but are expensive and generate concentrated wastes that require subsequent disposal (Lykins and Clark 1994; Pontius 1995; Kapoor et al. 1995; Srivastava et al. 1986; Komori et al. 1990). Biological reduction of selenate and chromate may provide a superior treatment alternative because selenate and chromate are oxidized forms that can be reduced to less toxic and less mobile species (Lovley and Coates 1997). Bacterial reduction of Se(VI) to elemental Se (Se^0) via Se (IV) is a feasible technology (Maiers et al. 1988; Losi and Frankenberger 1997a; Kashiwa et al. 2000); because Se^0 is insoluble, it can be removed by filtration or centrifugation, and has low toxicity (Maiers et al. 1988; Kashiwa et al. 2000; Oremland et al. 1994). Biological reduction also may provide a suitable means for Cr(VI) removal from water and wastewater (Lovley and Coates 1997; Rittmann et al. 2004), because Cr(VI) is reduced to Cr(III), which can be precipitated as $Cr(OH)_{3(s)}$ and removed by filtration. Cr (VI) is bioreduced to Cr(III) under aerobic (Bopp and Ehrlich 1988; Gopalan and Veeramani 1994) and anaerobic conditions (Lovley 1993; Wang et al. 1989).

Selenate-reducing bacteria have been isolated from many different aquatic environments (Cantafio et al. 1996; Barton et al. 1991, 1994; Francisco et al. 1992; Losi and Frankenberger 1997b; Oremland et al. 1999;

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Lortie et al. 1992): e.g., strain SES-3 was isolated from an estuarine sediment (Oremland et al. 1994), *Thauera selenatis* from Se(VI)-contaminated wastewater (Macy et al. 1989), *γ-proteobacteria* from solar evaporation pond salt (De Souza et al. 2001), and *Enterobacter taylorae* from rice straw (Zahir et al. 2003; Zhang et al. 2003).

Likewise, many microorganisms have been reported to reduce the highly soluble and toxic Cr(VI) to the less soluble and less toxic Cr(III), e.g., *Pseudomonas fluorescens* LB300 (Bopp and Ehrlich 1988), *Pseudomonas putida* (Park et al. 2000), *Enterobacter cloacae* HO1 (Wang et al. 1989), *Shewanella putrefaciens* MR-1 (Myers et al. 2000; Wang 2000), and *Bacillus* sp. (Wang and Xiao 1995). However, the potential for biological treatment of Cr(VI)-contaminated waste may be limited because some microorganisms lose viability in the presence of high concentrations of chromate (Cervantes and Ohtake 1988; Vonburg and Liu 1993). Some bacteria, such as *P. fluorescens* LB300, *P. aeruginosa*, *Alcaligenes eutrophus*, and *E. cloacae* HO1, have chromate resistance, but Cr(VI) reduction and Cr(VI) resistance have been considered unrelated (Ohtake et al. 1987), at least when chromate resistance is due to the presence of efflux mechanisms that allow resistant strains to extrude CrO_4^{2-} ions (Bopp and Ehrlich 1988; Cervantes and Ohtake 1988; Nies and Silver 1989; Ohtake et al. 1990).

The membrane biofilm reactor (MBfR), a new technical approach for bioreduction of oxidized contaminants (Ergas and Reuss 2001; Lee and Rittmann 2002; Nerenberg et al. 2002; Nerenberg and Rittmann 2004; Rittmann et al. 2004), uses hydrogen gas (H_2) as the electron donor. Hydrogen is nontoxic and leaves no residuals that could cause bacterial regrowth. Hydrogen also serves as a biologically available electron donor for the reduction of many oxidized contaminants (Nerenberg and Rittmann 2004; Rittmann et al. 2004).

In our previous studies, we evaluated the potential for reducing selenate to Se^0 and chromate to Cr(III) using the H_2-based MBfR (Chung et al. 2006a,b; in review). The MBfRs tested in these cases reduced Se(VI) to Se^0 and Cr (VI) to Cr(III) when applied separately (Chung et al. 2006a,b), and the same MBfRs reduced the other oxidized contaminant immediately when we applied Cr(VI) to the selenate-reducing MBfR and vice versa (Chung et al., in review). The significant reductions of both oxidized contaminants and the functional versatility of the biofilm communities in the two MBfRs make investigation of the microbial ecology of the biofilms of special interest. When the contaminant concentration is below threshold for sustaining the biomass that degrades it [i.e., its S_{min} (Rittmann and McCarty 1981; Namkung and Rittmann 1987)], it must be removed as secondary electron acceptor along with a primary acceptor that supports biomass (Nerenberg and Rittmann 2002a). Key questions are whether the primary acceptor will select for bacteria capable of utilizing the secondary acceptor, and, if not, whether the secondary acceptor can provide a selective pressure for those bacteria. These questions were addressed for MBfRs in which perchlorate was a secondary and nitrate a primary acceptor (Nerenberg and Rittmann 2002b; Nerenberg et al. 2003).

In this study, we investigate the microbial community of the two denitrifying MBfRs that showed selenate and chromate reductions. We correlate the reduction activities (i.e., functional diversity) in the MBfRs with community structure as determined by cloning.

Materials and methods

Experimental set-up and operating conditions

The schematics of MBfRs are shown in Fig. 1, and the MBfRs used in this study the same as described in Chung et al. (2006a,b). Briefly, each MBfR system consisted of two glass tubes connected with Norprene tubing and plastic barbed fittings. The interior of the fiber was connected to a pressurized H_2 supply at one end and sealed at the other end. Water circulated outside of the fiber, and H_2 diffused from the lumen of the fiber through the wall and into the biofilm, where it was oxidized. The membranes were produced by Mitsubishi Rayon (Model MHF 200TL), a composite bubbleless gas-transfer membrane with a total diameter of approximately 280 μm. Hydrophobic polyethylene with small (0.1 μm) pores comprised the inner and outer layers of the composite fiber. The fiber pores remained dry because the polyethylene is hydrophobic. A thin layer (~1 μm) of dense polyurethane was sandwiched between the polyethylene layer and blocked all pores polyethylene; thus, hydrogen molecules had to diffuse through the wall, making it a bubbleless gas-transfer device. Biofilm samples were collected by cutting short lengths of a separate "coupon" fiber, located in the second glass tube. This allowed sample collection without disturbing the main bundle of fibers and without causing a significant change in total biofilm surface area in the reactor.

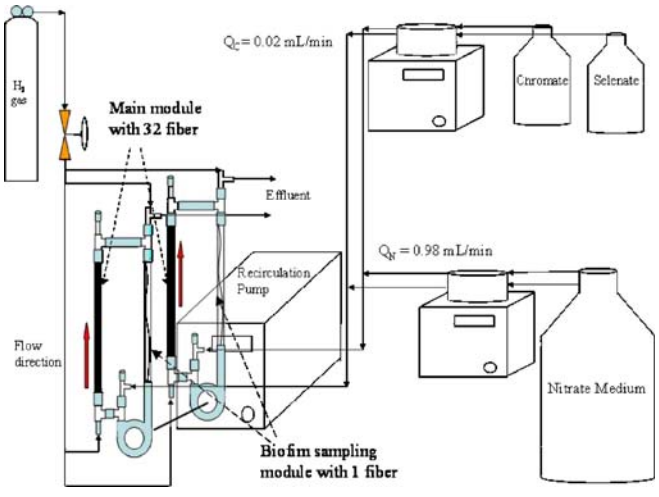

Fig. 1 Schematic of the bench-scale MBfRs used to investigate Se and Cr reduction

A single peristaltic manifold pump (Gilson Minipuls 3, Middleton, WI, USA) was used with PVC tubing to give a nitrate-medium-feed rate of 0.98 ml/min. A second manifold pump supplied the selenate and chromate stock solution at rate of 0.02 ml/min into the feed line from the nitrate medium, thereby giving a total feed flow rate of 1 ml/min. The recirculation rate was 150 ml/min, giving a recirculation ratio of 150 that promoted completely mixed conditions. Another advantage of a high-recirculation rate was the high-hydraulic shear on the fibers, providing a dense biofilm (Chang et al. 1991) that minimized the accumulation of excessive biofilm that might clog the reactor. The H_2 pressure for both MBfRs was 2.5 psi (0.17 atm) though all experimental periods.

The composition of the main feed medium was (g/l): KH_2PO_4 0.128, 100, Na_2HPO_4 0.434, $MgSO_4 \cdot 7H_2O$ 0.2, $NaNO_3$ as N 0.03, $CaCl_2 \cdot 2H_2O$ 0.001, $FeSO_4 \cdot 7H_2O$ 0.001, and 1 ml of trace mineral solution. The trace mineral solution (mg/l) consisted of $ZnSO_4 \cdot 7H_2O$ 100, $MnCl_2 \cdot 4H_2O$ 30, H_3BO_3 300, $CoCl_2 \cdot 6H_2O$ 200, $CuCl_2 \cdot 2H_2O$ 10, NiCl2•$6H_2O$ 10, $Na_2MoO_4 \cdot 2H_2O$ 30, and Na_2SeO_3 30. The nominal influent concentrations of potential electron acceptors were 5 mg $NO_3^- - N/l$, 78.5 mg SO_4^{2-}/l, and 250 μg/l as Se or Cr. Actual concentrations were measured daily. All feed media were purged with nitrogen gas to eliminate dissolved O2 in the influent. The inoculum was obtained from a pilot-scale MBfR operated at La Puente, California (Rittmann et al. 2004), frozen in glycerol, thawed, washed, resuspended, and added to the MBfRs, as detailed in Chung et al. (2006a).

Start up began when H_2 was supplied to the membrane, and the liquid in the reactor was recirculated for 24 h to establish a biofilm. Then, influent medium that contained no chromate or selenate was fed at a rate of 0.2 ml/min. The concentration of nitrate (5 mg-N/l) in the effluent reached steady state after around 3 days, and then the feed rate of influent medium was increased to 1.0 ml/min. After nitrate was completely reduced in the effluent from MBfR (c. 20 days), selenate or chromate was added to the influent medium (5 mg-N/l of nitrate) of each MBfR at 250 μg/l as Se or Cr. After approximately 50 days, the oxidized contaminants to each MBfR were switched.

Liquid sampling and analysis

The performance of the MBfRs was monitored by analyzing influent and effluent samples for soluble nitrate, nitrite, chromate, Cr(III), Se(VI), Se(IV), produced Se^0, sulfate, and sulfide according to standard procedures that are described in detail by Chung et al. (2006a,b).

Scanning electron microscopy and energy dispersive X-ray spectroscopy analysis

A control membrane fiber was cleaned and its surface examined by scanning electron microscopy (SEM) directly after washing with 1 M NaOH and sonication for 30 min. Biofilm samples taken as 2–3 cm sections of fiber from the single fiber in the two MBfRs were examined by SEM on day 42, when MBfR A was reducing selenate and MBfR B was reducing chromate. Sample preparation for SEM followed a modification of the procedure described in Sutton et al. (1994). A sample of hollow-fiber membrane was immersed in 1% (*v*/*v*) glutaraldehyde solution for 2 h and then rinsed with 16-mM phosphate buffer at pH 7.1. The sample was dehydrated in increasing concentrations of ethanol for 15 min each (once in 10, 30, 50, 70, and 90%, and twice in 100%). The ethanol was replaced with liquid CO_2 using a Polaron Critical Point Drier (Quorum Technologies, East Sussex, England). Finally, the samples were sputter coated with Au/Pd at a 3-nm thickness using a Cressington 208HR sputter coater (Cranberry Twp., PA, USA) to enhance the quality of the image. Noncoated samples showed lower resolution and uneven brightness (images not shown). All samples were analyzed at a voltage of 15 kV and an average working distance of 13.5± 2.3 mm under a secondary electron (SE) detector and a backscattered electron detector.

Elemental compositions of the insoluble precipitate observed in Se-reducing and Cr-reducing biofilms were determined using energy dispersive X-ray spectroscopy (EDS; Hitachi S-3500N VP-SEM-EDS) according to the manufacturer's instructions. Samples were analyzed using point and scan analysis for at least 100 s.

Biofilm sampling and DNA amplification

Three biofilm samples were collected for molecular analyses on days 0, 42, and 80 after nitrate was completely reduced. Biofilm samples were taken as 2- to 3-cm sections from the single fiber in the coupon tube of each MBfR. DNA was extracted from the biofilm sample using the Ultra Clean Soil DNA kit (MoBio Laboratories, Solana Beach, CA, USA), which includes bead-beating and a spin-column purification steps (Tsai and Rochelle 2001). Successful extraction was confirmed by gel electrophoresis, and DNA was stored at −20 °C until further processed.

The 16S rDNA genes from extracted biofilm samples were amplified by the polymerase chain reaction (PCR) using PCR Master (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. The primer set was 1070f (forward primer; 5′-ATGGCTGTC GTCAGCT-3′) and 1392r (reverse primer; 5′-ACGGGCG GTGTGTAC-3′). These primers amplify a 322-bp section of the gene of members of the domain bacteria including the highly variable V9 region (Gray et al. 1984). PCR was performed using a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA). Amplification conditions were as follows: preincubation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C, annealing at 55 °C, extension at 72 °C each for 45 s, a single final extension at 72 °C for 10 min, and incubation at 4 °C until processed further. Amplification products were separated by horizontal gel electrophoresis on a 1.6% agarose gel (Amresco, Solon, OH, USA). The agarose gels were

stained with ethidium bromide (Sigma Chemical, St. Louis, MO, USA) and visualized under UV light. Gel images were captured using a gel documentation system (UVP, Upland, CA, USA).

Cloning and DNA sequencing analyses

Amplicons were purified with a QIAquick PCR purification kit (Qiagen Sciences, Valencia, CA, USA) and ligated into pCR4 TOPO plasmid vectors (TOPO TA cloning kits, Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions for maximum plasmid yields. Ligated vectors were transformed into chemically competent *Escherichia coli* cells and grown overnight at 37 °C on Luria–Bertani (LB) agar medium (Mikrobiologie, EM Science, Gibbstown, NJ, USA) amended with 100 μg of ampicillin per milligram. Colonies were screened for inserts from PCR with plasmid-vector specific primers T3 (5′-ATT AAC CCT CAC TAA AGG GA-3′) and T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′). Positive clones produced a 482-bp PCR product containing the 322-bp small-subunit rRNA insert and the two 80-bp flanking regions of the plasmid vector. The percent similarity to reference strains found in the GenBank database was estimated within a 322-bp section of the gene. After enzymatic removal of unincorporated primers and deoxynucleoside triphosphates by using ExoSAP-IT (USB, Cleveland, OH, USA), confirmed plasmid inserts were directly sequenced using an automated ABI Prism 377 DNA sequencer with a Big Dye sequencing kit (Applied Biosystems, Foster City, CA, USA) with 5 μl (ca. 500 ng) of the purified plasmid DNA with 20 ng of the plasmid-specific T3 and T7 primers. Retrieved sequences were compared with available sequences with the National Center for Biotechnology Information BLAST program (Altschul et al. 1990). Sequences were aligned with ClustalX software (Thompson et al. 1994). The Phylip software package was used for construction of phylogenetic trees by the neighbor-joining method and for construction of similarity matrixes (Felsenstein 1999; Saitiu and Nei 1987).

Results

Function of the MBfRs

In the first few days of operation after start up in both MBfRs, nitrate was partially converted to nitrite, but the nitrate and nitrite concentrations in the effluent dropped to less than 15 μg-N/l within 10 days. For the steady-state reduction of nitrate, the average removal of nitrate was 99± 1%. Sulfate reduction in both MBfRs began within 5 days. Neither sulfate nor sulfide inhibited nitrate or nitrite reduction. Feeding of Se(VI) or Cr(VI) began 20 days after start up, or 10 days after nitrate and nitrite were less than 15 μg-N/l. For the results presented below, day 0 refers to the first day on which Se(VI) or Cr(VI) was added to the influent.

Figure 2 shows the concentrations of Se(VI), Se(IV), total selenium, produced elemental selenium, Cr(VI), Cr (III), and total Cr in the effluent from MBfR A once Se(VI) and (later) Cr(VI) feeding began. Se(VI) reduction to Se (IV) and Se^0 began within 1 day of Se(VI) addition, and Se (VI) and Se(IV) were almost completely reduced to Se^0 (243 μg-Se/l, or 97%) by day 20 of Se(VI) feeding. The Se^0 formed was either retained in the biofilm or in a solid form removed by filtration with a 0.2-μm membrane filter because total selenium in the effluent was only 4.7 μg/l, or 2% of the influent. The immediate onset of Se(VI) reduction suggests that selenate-reducing bacteria were present in the biofilm in significant numbers. The steady increase in Se(VI) reduction suggests that continuous exposure to Se(VI) provided a selective pressure that enriched dissimilatory selenate-reducing bacteria over about 20 days.

Figure 2 also shows that, when the oxidized contaminant was switched from Se(VI) to Cr(VI) (day 50), Cr(VI) was immediately reduced to Cr(III): 68% removal of Cr(VI) on the first day. By day 60 (or 10 days after switching feed of

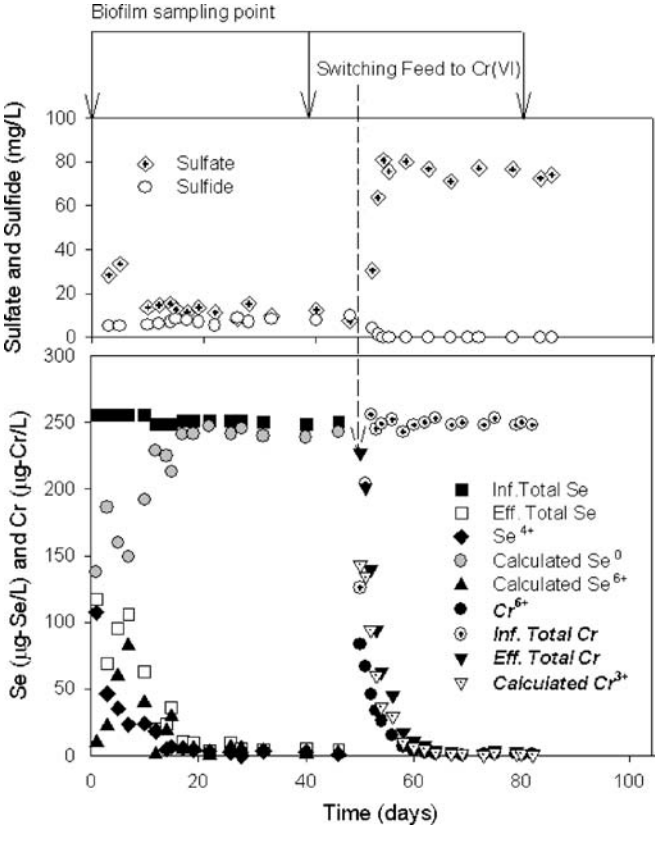

Fig. 2 Concentrations of chromium and selenium species in effluent from MBfR A. Up to day 49, 5 mg/l of NO_3^- N and 250 μg-Se/l were in the influent. From day 50, 5 mg/l of NO_3^- N and 250 μg-Cr/l were in the influent. *Upper left Y-axis* sulfate and sulfide in mg/l and *lower left Y-axis* Se species in μg-Se/l and Cr species in μg-Cr/l. The *solid arrows* indicate the biofilm sampling point for cloning, SEM, and EDS

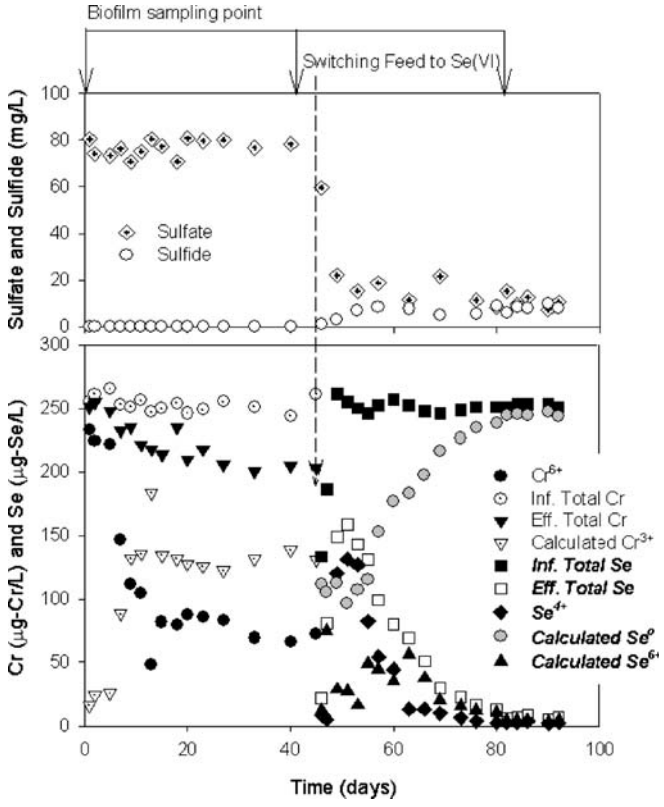

Fig. 3 Concentrations of chromium and selenium species in effluent from MBfR B. Up to day 45, 5 mg/l of NO_3^-N and 250 μg-Cr/l were in the influent. From day 46, 5 mg/l of NO_3^-N and 250 μg-Se/l were in the influent. *Upper left Y-axis* sulfate and sulfide in mg/l and *lower left Y-axis* Se species in μg-Se/l and Cr species in μg-Cr/l). The *solid arrows* indicate the biofilm sampling point for cloning, SEM, and EDS

Cr(VI) begun), most of the Cr(VI) was reduced to Cr(III), and the Cr(III) species must have been precipitated as $Cr(OH)_{3(s)}$ and removed in the biofilm or by filtration: By 20 days, total chromium in the filtered effluent was below 10 μg-Cr/l.

Figure 3 summarizes the results for MBfR B, which was fed Cr(VI) first at 250 μg-Cr/l. The reduction of Cr(VI) to Cr(III) began immediately, indicated by 25 μg-Cr/l of Cr (III) in the 1-day sample after chromate addition. Steady-state reduction of Cr(VI) to Cr(III) was evident after 18 days, and the average reduction of Cr(VI) to Cr(III) was 65±6%, yielding an effluent Cr(III) concentration of 123± 18 μg-Cr/l. The effluent concentration of total Cr reached approximately 211 μg-Cr/l, of which Cr(III) was 123 μg-Cr/l. Thus, Cr(III) either was soluble or was a colloid that was not removed by filtration and centrifugation of the effluent.

Figure 3 also presents the concentrations of selenium species in the effluent from MBfR B after 250 μg-Se/l was applied in the influent beginning on day 52. As soon as Se (VI) feeding began, Se(VI) was reduced to Se(IV) and Se^0. By day 69, or 17 days after switching the feed to Se(VI), the concentrations of Se (IV) and Se (VI) were below 20 μg-Se/l, making the effluent total Se less than 29 μg-Se/l and showing that most of the selenate had been reduced to Se^0, which was removed in the biofilm or by effluent filtration. The Se(VI) flux and the electron-equivalent flux for Se(VI) were 0.036 g Se(VI)/m^2 of biofilm surface area/day and 0.47 e^-meq/m^2 per day, respectively. Also, electron-equivalent fluxes for nitrate and sulfate were 58 and 51 e^- meq/m^2 per day, respectively. In this case, the sulfate flux did not increase once chromate was removed from the influent.

Electron microscopic and energy-dispersive X-ray analyses of biofilm from both MBfRs

Figure 4 shows the surface morphology of a control hollow fiber (left) and the biofilm surface from MBfR A (middle) and MBfR B (right). The biofilm samples were taken on day 42, when MBfR A was reducing selenate and MBfR B was reducing chromate. The clean control fiber shows the microporous structure of the fiber's outside polyethylene layer; the surface is flat with a relatively uniform pores of 0.1 to 0.3 μm. The biofilm MBfR B consisted of individual rod-shaped bacteria about 0.3 μm in diameter and 2 μm long. A significant amount of inorganic precipitate accumulated at the surface of the cells in MBfR A, as is identified by the arrow in Fig. 4 (middle). Mineral precipitate also accumulated in the biofilm of MBfR B, but was not so easily revealed.

EDS analysis of the precipitate region in the biofilm sample from MBfR A and MBfR B at V_a=15.0 kV was employed to determine whether Se or Cr was a major component in the inorganic solids within the biofilm. As shown in Fig. 5a, EDS peaks for Se, Fe, and S appeared for the biofilm in MBfR A. On the other hand, the EDS

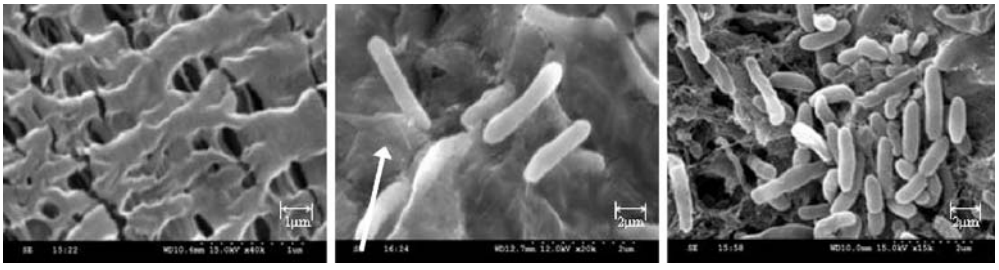

Fig. 4 Scanning electron micrograph of control fiber (*left*), biofilm from MBfR A (*middle*), and biofilm from MBfR B (*right*). The biofilm samples were taken on day 42. The images were taken using an SE detector at 15 kV and average 10.3 mm WD. The *arrow* points to an example of an inorganic precipitate

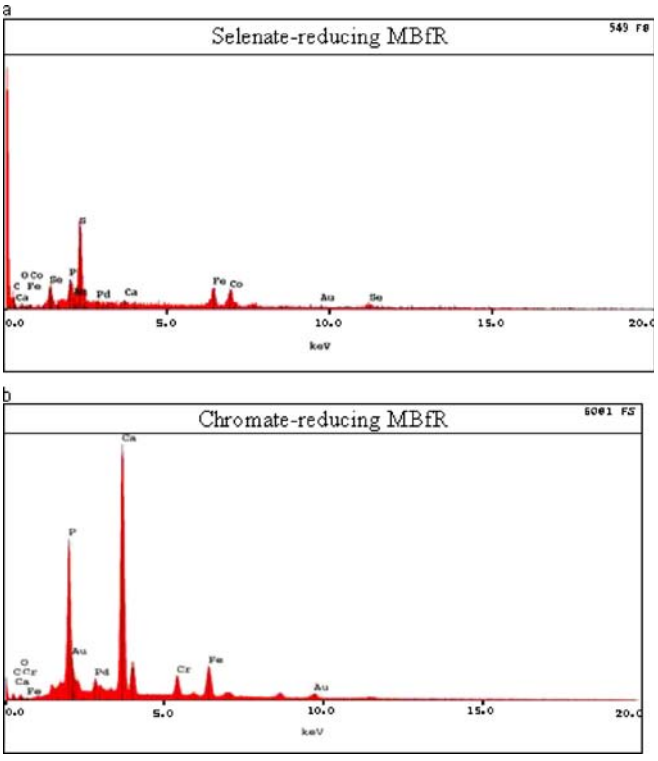

Fig. 5 EDS obtained from the insoluble precipitate formed the biofilm from **a** MBfR A and **b** MBfR B. Biofilm samples were taken on day 42. Similar spectra were obtained (not shown) from analyzing the region at a higher magnification

spectrum in Fig. 5b for MBfR B shows a pronounced Cr and Fe peak.

Identification of community structure using cloning

The phylogenetic structure of each MBfR's biofilm community was investigated using direct cloning and sequencing of the PCR-amplified V9 region of the 16S rRNA gene from the domain *Bacteria*. Nucleotide sequences were determined for 128 clones from the two MBfRs. BLASTn analysis was performed to examine the similarity of the clones to reference strains found in the GenBank databases. All the nucleotide sequences of the clones showed >99% similarity to one of the reference strains (Table 1). The distribution of clones from each MBfR sample among the six reference strains is listed in the Table 1.

In MBfR A, PCRB (i.e., *Dechloromonas* and *Dechlorosoma*) were the dominant strains, but declined in relative fractions of clones (from 80 to 40%) as the MBfR received selenate and then chromate. As the PCRB declined, *Pseudomonas* strains increased to 40% of the clones when Cr(VI) was added to the system. *Pseudomonas* species are well known as denitrifiers (Samuelsson 1985; Härtig et al. 1999), but not sulfate reducers. The genus *Pseudomomas* is well known for its metabolic diversity with respect to organic electron donors, but not for a wide diversity of electron acceptors apart from nitrate and oxygen.

In MBfR B, the PCRB were stable as the dominant group, having between 70 and 74% of the clones from the time before chromate was added, to when chromate was reduced, and finally when selenate was reduced. In MBfR B, the fraction of *Pseudomonas* spp. increased by 10%, but its fraction of the clones did not increase after switching influent feed to Se(VI).

Discussion

For the results shown in Fig. 2 for MBfR A, the Se(VI) flux was 0.034 g Se(VI)/m^2 of membrane surface area/day, and nitrate and sulfate fluxes were $0.71g-NO_3^-/m^2$ per day and $7.9g-SO_4^{2-}/m^2$ per day , respectively. The electron-

Table 1 Frequencies of dominant bacterial strains from two MBfRs and their phylogenetic affiliations

Clone type	Phylogenetic relationship			MBfR A			MBfR B		
	Species	Accession no.	Similarity (%)	Day 0, initial community	Day 42, Se(VI) reduction	Day 80, Cr(VI) reduction	Day 0, initial community	Day 42, Cr(VI) reduction	Day 80, Se(VI) reduction
A	*Dechloromonas denitrificans*	AJ318917	100	70%	42%	40%	70%	40%	58%
B	*Dechloromonas spp.*	AY515723	99	10%	<4%	<5%	<5%	30%	16%
C	*Dechlorosoma*	AY126453	99	<5%	8%	<5%	<5%	<5%	<4%
A+B+C	Perchlorate Reducing Bacteria			80%	50%	40%	70%	70%	74%
D	*Acinetobacter* spp.	AF542963	100	<5%	4%	<5%	20%	10%	<4%
E	*Pseudomonas* spp.	D87103	99	<5%	29%	40%	<5%	10%	<4%
F	*Rhodanobacter* spp.	AF494542	99	10%	<4%	10%	<5%	<5%	8%
Total clones				20	24	20	20	20	24

Percent similarity to reference strains found in the GenBank database was estimated within a 322-bp section of the gene of members of the domain bacteria including the highly variable V9 region

equivalent flux for Se(VI) was 0.48 e^- meq/m^2 per day, which is much smaller than the electron-equivalent fluxes for nitrate and sulfate, 56 and 710 e^- meq/m^2 per day, respectively. This result means that sulfate reduction dominated the electron-equivalent flux so that the total demand for H_2 was mostly controlled by sulfate reduction, not selenate reduction. The Cr(VI) flux in MBfR A was 0.031 g-Cr(VI)/m^2 per day, and its electron-equivalent flux was 0.80 e^- meq/m^2 per day. Also, electron-equivalent fluxes for nitrate and sulfate were 57 and 49 e^- meq/m^2 per day, respectively. The significantly reduced flux of sulfate suggests that chromium was inhibitory to sulfate reduction, a phenomenon we observed in a previous study with chromate (Chung et al. 2006b).

The Cr(VI) flux in MBfR B (Fig. 3) was 0.022 g Cr(VI)/m^2 of biofilm surface area/day. Converted to common e^- eq units, Cr(VI), nitrate, and sulfate fluxes were 0.54, 67, and 46 e^- mEq/m^2 per day, respectively. Thus, nitrate and sulfate fluxes together accounted for 99.5% of the total electron flux, indicating that the total demand for H_2 was not controlled by Cr(VI) reduction. Similar to MBfR A when chromate was applied, the nitrate and sulfate fluxes were similar, and sulfate reduction was seldom observed. After applying Se(VI) in the influent, the Se(VI) flux and the electron-equivalent flux for Se(VI), nitrate, and sulfate were similar to those in MBfR A fed with Se(VI). The bioactivity reduction for both contaminants in the two MBfRs was similar.

Although it is generally thought that Cr(III) is immobile within pH values of about 5.5–11, due to $Cr(OH)_{3(s)}$ precipitation, the degree of immobilization depends on how the conditional solubility compares to the target Cr (III) concentration (Ziemniak et al. 1998; Cherry 1982). Chung et al. 2006b provide a detailed evaluation of the solubility of $Cr(OH)_{3(s)}$ in the medium used. In summary, the conditional solubility of Cr(III) calculated for pH 7.5 for this study is approximately 64 μg-Cr/l when hydroxide complexes are taken into account. Hence, the observed higher Cr(III) concentration in MBfR B, if truly soluble, probably was due to Cr(III) complexation with phosphate, sulfate, nitrate, fluoride, citrate, or soluble organic ligands (Evanko and Dzombak 1997; Armienta and Quere 1995; Rai et al. 2004). The most likely ligand of importance in the experimental medium was phosphate, which was present at a total concentration of 0.004 M. On the other hand, the concentration of Cr(III) in the effluent from MBfR A was much lower than from MBfR B and the conditional solubility of 64 μg-Cr(III)/l, even though the influent loading of Cr(VI) was the same. This suggests another sink for Cr(III) in MBfR A.

We confirmed at bench-scale that MBfRs have the potential to reduce selenate and chromate. A major difference between the bioreduction of selenate and chromate and the more familiar biological denitrification is that selenate and chromate typically are present at concentration orders of magnitudes lower than nitrate. Consequently, chromate and selenate may be reduced as secondary substrates, with another electron acceptor (nitrate and/or sulfate in this case) serving as the primary substrate, or the acceptor whose reduction generates most of the energy that the microorganisms use for synthesis and maintenance. Nerenberg et al. (2002) saw the secondary-utilization effect when small concentrations of perchlorate were reduced concurrently with larger concentration of nitrate in an MBfR. Secondary utilization makes it possible to achieve very low effluent concentrations of the secondary substrate because ample biomass is sustained by utilization of the primary substrate.

An EDS peak for Se appeared for the biofilm in MBfR A, demonstrating that Se^0 was accumulating in the biofilm. The more pronounced S peak indicates higher reduction of sulfate to form sulfide solids, particularly FeS. On the other hand, the EDS spectrum for MBfR B shows a pronounced Cr peak, comparable to the Fe peak, confirming that Cr solid was present in the biofilm of MBfR B. For MBfR B, P, and Ca peaks also are pronounced. Thus, the inorganic phase in the biofilm likely also contained a calcium phosphate solid, a finding noted before by Lee and Rittmann (2003) for denitrification in MBfR.

The largest fraction of clones was closely related to known perchlorate-reducing bacteria (PCRB) in the *Dechloromonas* and *Dechlorosomas* genera, with *Dechloromonas denitrificans* being the most common. Nitrate and sulfate were reduced concurrently in MBfRs A and B, with electron-equivalent fluxes much larger than for Cr(VI) or Se(VI); sulfate had a much larger electron-equivalent flux at the time of first biofilm sampling of MBfR A, but the fluxes were equal for other biofilm samples. The relatively large fluxes of nitrate and sulfate, the similar community structures, and the immediate reductions of chromate and selenate suggest that Cr(VI) and Se(VI) were reduced as secondary acceptors, while nitrate, sulfate, or both were the primary electron acceptors (Nerenberg and Rittmann 2002a; Nerenberg et al. 2003).

Previous studies on the ubiquity and diversity of PCRB described *Dechloromonas* and *Dechlorosoma* species, which appear to be the dominant PCRB in the environment (Coates et al. 1999a; Achenbach et al. 2001; Wolterink et al. 2005). (Per)chlorate-reducing activity has been observed not only in chlorate- or perchlorate-polluted samples, but also in pristine sediments and soils (van Ginkel et al. 1995; Coates et al. 1999a; Wu et al. 2001). Also, *Dechloromonas denitrificans* can reduce sulfate, nitrate, and nitrite (Horn et al. 2005), and *Dechloromonas aromatica* strain RCB was the only organism in pure culture able to oxidize benzene in the absence of oxygen (Coates et al. 2001). *D. aromatica* also can oxidize other aromatics such as toluene, benzoate, and chlorobenzoate. *D. aromatica* couples growth and benzene oxidation to the reduction of either O_2, chlorate, or nitrate (Coates et al. 2001). Nerenberg et al. (2002) isolated a H_2-oxidizing strain of *Dechloromonas*, *D.* PC1, that was important in MBfRs reducing perchlorate and used nitrate or oxygen as its primary acceptor. In summary, *Dechloromonas* is a unique genus with a broad range of novel metabolic capabilities and bioremediative applicability (Coates et al. 1999b, 2001) in terms of electron acceptors and donors. Thus, it is not surprising that *Dechloromonas* strains are

predominant in the biofilms of MBfRs A and B. What is new here is its apparent ability to reduce Cr(VI) and Se(VI).

The most likely explanation for the significant presence of *Pseudomonas* in MBfR A may be that they were growing by oxidizing soluble microbial products (De Silva and Rittmann 2000; Laspidou and Rittmann 2002) released by the autotrophic H_2 oxidizers and through the reduction of nitrate. Based on the known characteristics of *Pseudomonas* and the lack of consistent correlation of them with chromate or selenate reduction, the results from this study suggest that they were not involved in chromate and selenate reduction. To the contrary, the PCRB probably were the main chromate- and selenate-reducing bacteria.

The cloning and sequencing method used in this study is a useful screening tool to identify dominant strains in unknown microbial community. However, it is limited in quantification of relative distribution of microbial community in biofilms. Future attempts to analyze the absolute distribution of bacterial strains in mixed microbial communities need to focus in a few distinct bacterial strains and apply quantitative techniques for these stains. An alternative method to estimate absolute numbers of the cells is to study the number of rRNA copies per genome of the bacterial cells. For MBfR study, *Dechloromonas* spp. and *Pseudomonas* spp. appear to be high priority targets to be investigated intensively using quantitative real-time PCR, slot-blot hybridization, or fluorescent in situ hybridization, which provide important information that complements direct cloning and sequencing method. Given the known metabolic versatility of the perchlorate-reducing bacteria and their dominance in the MBfR biofilms, the perchlorate-reducing bacteria—particularly *Dechloromonas* spp.—probably were responsible for selenate and chromate reductions. Our results support that H_2-oxidizing *Dechloromonas* sp. have the capability to reduce Cr(VI) and Se (VI) as secondary electron acceptors.

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