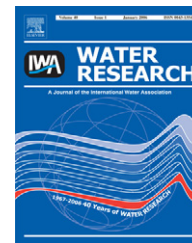


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# Microbial ecology of a perchlorate-reducing, hydrogen-based membrane biofilm reactor

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## ABSTRACT

The hydrogen-based membrane biofilm reactor (MBfR) has been shown to reduce perchlorate to below 4 µg/L, but little is known about the microbial ecology of this or other hydrogen-based reactors, especially when influent perchlorate concentrations are much lower than the influent oxygen and nitrate concentrations. Dissimilatory (per)chlorate-reducing bacteria (PCRB) can use oxygen as an electron acceptor, and most can also use nitrate. Since oxygen and nitrate can be reduced concurrently with perchlorate, they may serve as primary electron acceptors, sustaining PCRB when the perchlorate concentrations are very low. We studied five identical MBfRs, all seeded with the same inoculum and initially supplied with oxygen, or oxygen plus nitrate, in the influent. After 20 days, perchlorate was added to four MBfRs at influent concentrations of 100–10,000 µg/L, while the fifth was maintained as a control. One day after perchlorate addition, the MBfRs displayed limited perchlorate reduction, suggesting a low initial abundance of PCRB. However, perchlorate reduction improved significantly over time, and denaturing gradient gel electrophoresis (DGGE) analyses suggested an increasing abundance of a single *Dechloromonas* species. Fluorescence *in-situ* hybridization (FISH) tests showed that the *Dechloromonas* species accounted for 14% of the bacterial count in the control MBfR, and 22%, 31%, and 49% in the MBfRs receiving nitrate plus 100, 1000, and 10,000 µg/L perchlorate, respectively. The abundance was 34% in the MBfR receiving oxygen plus 1000 µg/L perchlorate. These results suggest that oxygen is more favorable than nitrate as a primary electron acceptor for PCRB, that PCRB are present at low levels even without perchlorate, and that the presence of perchlorate, even at low levels relative to nitrate or oxygen, significantly enhances selection for PCRB.

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## 1. Introduction

Perchlorate (ClO<sub>4</sub><sup>-</sup>) contamination of surface and ground waters has become a significant environmental concern, with

detections throughout the United States (Gullick et al., 2001). Although perchlorate is used in a wide range of industrial applications (Kirk et al., 1991), the contamination has mainly been attributed to two sources: defense-related facilities,

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where synthetically manufactured ammonium perchlorate was used in rocket fuels (Motzer, 2001; Wallace et al., 1996), and fertilizers made with perchlorate-containing nitrates imported from Chile (Ericksen, 1983; Schilt, 1979). Recent studies show that perchlorate is present in rain and snow, suggesting that it is naturally formed in the atmosphere (Dasgupta et al., 2005; Rao et al., 2007). Perchlorate is a concern due to its inhibition of thyroid function (Clark, 2000; Stanbury and Wyngaarden, 1952). It is on the US Contaminant Candidate List (Scharfenaker, 2005), and a recently published reference dose of 0.7 mg/kg day translates to a drinking-water equivalent level of 24.5 µg/L if water were the only source of perchlorate exposure (Dahl, 2005; Ginsberg and Rice, 2005). Several states have perchlorate advisory levels for drinking water at 6 µg/L or less (EPA, 2005).

Perchlorate is very soluble and stable in water, making it difficult to remove by conventional water-treatment processes (Schilt, 1979; Urbansky, 1998; Xu et al., 2003). However, biological reduction is a promising treatment approach (Hatzinger, 2005; Urbansky and Schock, 1999; Xu et al., 2003), as perchlorate can be reduced to chloride and water by dissimilatory (per)chlorate-reducing bacteria (PCRB), i.e., bacteria that reduce perchlorate and chlorate ( $\text{ClO}_3^-$ ) as electron acceptors that provide energy for growth (Coates and Achenbach, 2004; Xu et al., 2003). PCRB are ubiquitous in the environment, and perchlorate is highly energetic, with a redox potential similar to that of nitrate (Schilt, 1979). Perchlorate's reduction pathway is believed to include two specialized enzymes, (per)chlorate reductase and chlorite ( $\text{ClO}_2^-$ ) dismutase (Bender et al., 2002, 2005; Bruce et al., 1999; Coates and Achenbach, 2004; Kengen et al., 1999; Nerenberg et al., 2006; van Ginkel et al., 1996). The first reduces perchlorate to chlorate, and then chlorate to chlorite, with a total transfer of four electrons. The second transforms chlorite into chloride and oxygen via disproportionation. The produced oxygen is subsequently reduced via the conventional pathway with a transfer of four more electrons. All PCRB are facultative aerobes or microaerophiles (Coates and Achenbach, 2004). Although oxygen at high concentrations inhibits perchlorate reduction (Chaudhuri et al., 2002), all PCRB reduce oxygen produced from perchlorate reduction concurrently with perchlorate, and perchlorate reduction has been found to occur with measurable oxygen concentrations (Shrout and Parkin, 2006). Most PCRB can also use nitrate as an electron acceptor, although in most cases nitrate inhibits perchlorate reduction when its concentration is high enough (Chaudhuri et al., 2002; Coates et al., 1999a; Giblin and Frankenberger, 2001; Wallace et al., 1996). Chlorite also inhibits perchlorate reduction (Nerenberg et al., 2006).

In most water-treatment applications, perchlorate concentrations in the influent water are in the µg/L range, while oxygen and nitrate are in the mg/L range. On the one hand, respiration of nitrate and oxygen may select for non-PCRB, since it is not clear whether respiration of trace levels of perchlorate can exert a sufficient selective pressure for PCRB. On the other hand, nitrate and oxygen respirations may be beneficial to the perchlorate-reducing processes when they act as primary substrates that support PCRB when the perchlorate concentration is low or below the growth threshold (Nerenberg et al., 2006). In these cases, perchlorate acts as

a secondary substrate and is utilized concurrently with oxygen and nitrate (Namkung and Rittmann, 1987; Rittmann et al., 1994). Thus, the ways in which nitrate and oxygen respirations affect the microbial ecology of PCRB are of the utmost importance when perchlorate reduction is the goal.

Some ecology tests have been carried out on heterotrophic perchlorate-reducing bioreactors. Zhang et al. (2005) explored the abundance and spatial distribution of *Dechloromonas* and *Dechlorosoma* species in a pilot-scale packed-bed reactor supplied with 50–120 mg/L perchlorate, 9 mg/L dissolved oxygen, and 4 mgN/L nitrate. Acetate was added as an electron donor. The reactor was operated under plug-flow conditions, with electron donor and acceptor concentrations varying with bed depth. Using ribosomal intergenic spacer analysis (RISA) and fluorescence in-situ hybridization (FISH), they found that *Dechloromonas* and *Dechlorosoma* species accounted for less than 5% of total bacteria.

Nerenberg et al. (2002) studied a novel, hydrogen-based membrane biofilm reactor (MBfR), which reduced influent perchlorate concentrations of up to 1000 µg/L to below 4 µg/L. The reactor influent contained 5 mgN/L nitrate. In these studies, the addition of 1 mg/L perchlorate to a denitrifying MBfR resulted in an initial perchlorate reduction of around 40%, and then a gradual increase to 99% over 2 weeks. These results suggest that some PCRB were present under denitrifying conditions, but that adding perchlorate to the influent increased their abundance.

Our objective is to understand how and why PCRB become members of a microbial community of an MBfR biofilm when perchlorate concentrations are low. This understanding is at the core of understanding the microbial ecology in hydrogen-based biofilms. The understanding also is key for reliable MBfR operation. Closely related is the question of whether nitrate is required as a primary acceptor for trace perchlorate reduction, or if oxygen can also serve as a primary acceptor. In this research, we study the microbial ecology of a hydrogen-based, autotrophic bioreactor, with special emphasis on the role of oxygen and nitrate in selecting for perchlorate-reducing species.

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## 2. Methods

Five bench-scale, hydrogen-based MBfRs, designated R1–R5, were used to study the effect of perchlorate on the microbial ecology of a mixed-culture biofilm. Reactors R1–R4 had oxygen plus nitrate as primary electron acceptors, while R5 had only oxygen. The MBfRs were identical in physical configuration, and the experiments were conducted concurrently. Hydrogen was supplied in excess to prevent it from becoming rate limiting. The main focus of this research was to understand the effect of perchlorate at low influent concentrations. Higher influent perchlorate concentrations were included to allow its potentially subtle effects on the microbial ecology to be more clearly visualized.

### 2.1. Reactor configuration

Details of the MBfR configuration are described by Chung et al. (2006). Fig. 1 shows the configuration schematically, and

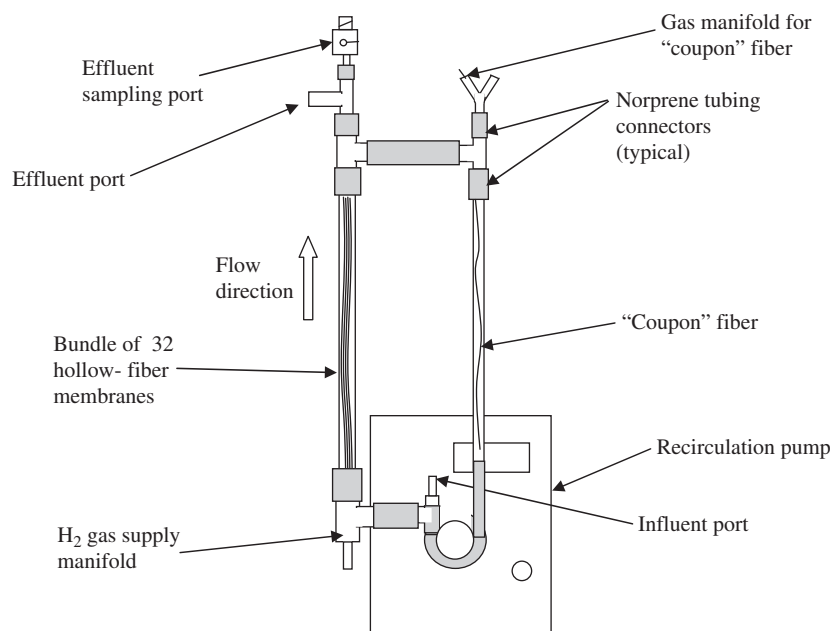


Fig. 1 – Schematic of the configuration of the bench-scale MBfR.

Table 1 – MBfR physical characteristics

	Units	Main tube	Coupon tube	Reactor total
Tube inside diameter	cm	0.6	0.5	–
No. of hollow fibers		32	1	33
Cross-sectional area of the fibers	cm <sup>2</sup>	0.0197	0.000616	0.0203
Net cross-sectional area for water flow	cm <sup>2</sup>	0.26	0.20	–
Fiber surface area	cm <sup>2</sup>	70.4	2.20	72.6
Liquid feed rate	mL/min	–	–	1 <sup>a</sup> or 0.85 <sup>b</sup>
Average detention time	min	–	–	23.9 <sup>a</sup> or 28.2 <sup>b</sup>
Recirculation rate	mL/min	–	–	150
Liquid velocity	cm/min	570	766	–

<sup>a</sup> Reactor 5.

<sup>b</sup> Reactors 1–4.

Table 1 summarizes the MBfR physical characteristics. Briefly, the MBfRs employed hydrophobic, microporous hollow-fiber membranes to deliver hydrogen gas to a biofilm naturally forming on the fibers' outer surface. Each MBfR consisted of two glass tubes connected with Norprene tubing and plastic fittings. One tube contained 32 hollow-fiber membranes, each with a 25-cm active length. The fibers were collected into a H<sub>2</sub>-supplying manifold at the bottom end, while the top of each fiber was sealed. A second tube contained a single 25-cm-long fiber that served as a sampling coupon for microbial ecology studies. The MBfR was completely mixed, due to the high recirculation rates; therefore, the concentrations in the MBfR were approximately equal to the effluent concentrations. The high recirculation velocity promotes biofilm detachment, which removes bacteria that are not active under the prevailing substrate conditions.

For reactors R1–R4, a single multi-channel manifold pump (Gilson Minipuls 3, Middleton, WI) was used to pump nitrate

plus oxygen medium from a 20-L polypropylene feed bottle to the four reactors. The pumping rate to each reactor was 0.98 mL/min. A second pump supplied stock solutions of perchlorate in deionized (DI) water at 0.5, 50, or 500 mg/L perchlorate at 0.02 mL/min into the feed lines for R1–R4. For R5, a single pump provided 1.0 mL/min from an 8-L glass feed bottle. On day 40, the flow to R1–R4 was reduced to 0.85 mL/min, with the flow proportions the same for R1–R4. The hydraulic retention time (HRT) for each reactor (Table 1) was confirmed with tracer tests prior to inoculation. The feed flow rates were monitored throughout the experiment and the manifold pump rates were adjusted as required.

## 2.2. Medium

The main medium included, per liter: 0.128 g KH<sub>2</sub>PO<sub>4</sub>, 0.434 g Na<sub>2</sub>HPO<sub>4</sub>, 0.2 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1 mg CaCl<sub>2</sub> · 2H<sub>2</sub>O, 1 mg FeSO<sub>4</sub> · 7H<sub>2</sub>O, 100 mg ZnSO<sub>4</sub> · 7H<sub>2</sub>O, 30 mg MnCl<sub>2</sub> · 4H<sub>2</sub>O, 300 mg H<sub>3</sub>BO<sub>3</sub>,

200 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 10 mg  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 10 mg  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 30 mg  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , and 30 mg  $\text{Na}_2\text{SeO}_3$ . The final pH was 7.4. For R1–R4, 5 mg N/L nitrate was added, and the dissolved oxygen (DO) was 6 mg/L. For R5, 20 mg/L  $(\text{NH}_4)_2\text{SO}_4$  was added as the nitrogen source, and the DO was 8 mg/L.

After reaching steady state with the main medium (20 days), reactors R1, R2, R3, and R4 were supplied with perchlorate at 0, 0.1, 1, and 10 mg/L, respectively, by amending the influent flow with perchlorate from stock solutions. For reactor R5, 1 mg/L perchlorate was added directly to its medium bottle at day 20.

### 2.3. Reactor startup and operation

The reactors initially were sterilized by adding several volumes of 6% hydrogen peroxide and recirculating it for 4 h. Then, the reactors were flushed with sterile medium and inoculated. The inoculum was obtained from a pilot-scale MBfR treating perchlorate-contaminated groundwater at La Puente, California (Adham et al., 2004). The groundwater fed to that reactor contained around 5 mgN/L nitrate and 60–90  $\mu\text{g/L}$  perchlorate, and the pilot MBfR achieved greater than 95% reduction of both. Following inoculation, the medium was recycled for 24 h to establish a biofilm. Influent medium was then supplied to each reactor as described above. The water temperature in the reactors was 25 °C.

### 2.4. Liquid-sampling and analytical methods

Influent and effluent samples were collected regularly and analyzed for dissolved components. The analytical methods for perchlorate, nitrate, liquid-phase hydrogen, pH, and dissolved oxygen were as described previously (Nerenberg et al., 2002), except that perchlorate was analyzed using a Dionex AS-11 column with an AG-11 pre-column with a 200- $\mu\text{L}$  injection loop. The perchlorate detection limit was 15  $\mu\text{g/L}$ .

### 2.5. Biofilm sampling

Biofilm samples were collected by cutting a 2-cm-long section from the coupon fiber. The remaining fiber was sealed by tying the end into a knot. The knot and fiber adjacent to the knot were discarded prior to collecting the 2-cm sample. Samples were collected on three occasions: after reaching steady state for nitrate in R1–R4 (day 20); after 16 days of perchlorate feed (day 36); and after 51 days of perchlorate feed (day 71). Biofilm samples were assayed by denaturing gradient gel electrophoresis (DGGE) to identify putative dominant strains and by FISH to quantify the dominant PCRb.

### 2.6. DGGE

DNA was extracted from biofilm samples and from isolate CH1 using the UltraClean Soil DNA Isolation Kit and the UltraClean Microbial DNA Isolation Kit (Mo Bio laboratories, Carlsbad, CA), respectively. The kits were used according to the manufacturer's instructions. The 16S rDNA was amplified by PCR using PCR Master kits (Roche Applied Science, Indianapolis, IN) and a thermal cycler (PCR Express, Hybaid, Ulm, Germany) with a "touchdown" PCR method (Giovannoni,

1991). The universal primer combination was GM5F-GC (forward primer) and DS907R (reverse primer) (Teske et al., 1996). The forward primer GM5F-GC contained a 40-base GC clamp. The amplified DNA fragment was around 550 base pairs (bp). DGGE was performed using a BioRad Dcode system (Bio Rad, Hercules, CA). A 6% (w/v) acrylamide gel was used and run in a 0.5  $\times$  TAE buffer. The denaturing gradient ranged from 30% to 60% denaturant (100% is 7 M urea and 40% (v/v) formamide). Electrophoresis was run at 75 V and 60 °C for 16 h. The gel then was stained with SYBR Green I (Molecular Probes, Eugene, OR) at 10,000  $\times$  dilution, and the gel's UV transillumination image was captured using a CCD camera system (Cohu Electronics model 4912-2010, San Diego, CA).

DGGE bands were excised with a sterile pipette tip and transferred to sterile 0.2-mL microcentrifuge tubes. After rinsing twice with 50  $\mu\text{L}$  of sterile, DI water, and eluting overnight at 4 °C, the suspended DNA was re-amplified by PCR as described above. The PCR products were purified using the UltraClean PCR Clean-up Kit (Mo Bio Laboratories, Carlsbad, CA) and sequenced using the ABI 3100 Prism BigDye terminator sequencing standard kit (Applied Biosystems, Foster City, CA) with the primer GM5F-GC or DS907R, according to the manufacturer's instructions. The partial sequence of 16S rDNA of isolate CH1 was amplified by PCR with the universal primer combination U27F and U1525R (Stahl and Amann, 1991), and the amplified DNA fragment was purified using the UltraClean PCR Clean-up Kit (Mo Bio Laboratories, Carlsbad, CA) and supplied for cycle sequencing. The cycle sequencing was performed, using the same procedure as described above, with seven universal primers: U27F, GM5F, U805F, U518R, U1093R, U1112F, and U1525R (Stahl and Amann, 1991). The DNA sequence was determined using an ABI 3100 DNA sequencer after purification of cycle sequence-product by a conventional ethanol-precipitation method. Nucleotide sequences were compared with known sequences in GenBank (Benson et al., 2002) using the BLAST program (Altschul et al., 1997).

### 2.7. FISH

Two oligonucleotide probes were designed, one for *Dechloromonas* CH1, a PCRb isolated from the MBfRs, and the second for PB9, an uncultured strain that was dominant in the DGGE gels. CH1 was isolated following a serial plating and re-growth method, as described previously (Nerenberg et al., 2006). The probes were designed with the PRIMROSE probe-design tool (Ashelford et al., 2002), with the 16S rRNA sequence of the *Dechloromonas* species enrolled in Ribosomal Database Project II (Cole et al., 2003). The probe sequence for CH1 was 5'-GCCCATGCGATTCTTCC-3', and it was 5'-ACCTATGCGATTCTTCCC-3' for PB9. The optimal formamide concentration (within the range of 20–55%) for hybridization was determined by applying each probe to a pure culture of CH1, and to the mixed-culture biofilm. When the formamide concentration was 40% or greater, probe PB9 did not hybridize with CH1 and the ratio of CH1-hybridized bacteria to others declined significantly. Below 40%, the ratio of CH1-hybridized bacteria and others in the mixed-culture biofilm was approximately constant. Therefore, 35% formamide was used for simultaneous FISH with both probes. The

biofilm was fixed with 1 mL of 4% formaldehyde solution in 1.5-ml microcentrifuge tubes for 18 h. Then, the formaldehyde solution was discarded, 1 mL of 1:1 mix of PBS/ethanol solution was added, and the specimen was preserved at  $-20^{\circ}\text{C}$ .

The biofilm was dispersed by sonication (Aquasonic model 250 HT, VWR Scientific, West Chester, PA), with periodic cooling on ice, for approximately 5 min and was mechanically disrupted by passing the specimen through a 200- $\mu\text{L}$  pipette tip. Then, a 20- $\mu\text{L}$  aliquot of sample was spotted on a gelatin-coated microscope slide and dehydrated with a 50%, 80%, and 96% ethanol series. After air-drying, 10  $\mu\text{L}$  of hybridization buffer (900 mM NaCl, 20 mM Tris/HCl, 35% formamide, 0.01% SDS) was applied, including 2 ng/ $\mu\text{L}$  of probe PB9 labeled with Cy3 (sulfoindocyanate) at the 5' end plus probe CH1 labeled with fluorescein isothiocyanate (FITC) at the 5' end.

Hybridization was performed at  $46^{\circ}\text{C}$  for 2 h, according to the procedure described previously (Manz et al., 1992; Pernthaler, 1997). After hybridization, the slide was immediately placed into 50 mL of pre-warmed washing buffer (52 mM NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS) and incubated at  $48^{\circ}\text{C}$  for 15 min. The slide was then washed with distilled water twice and air-dried. For the counter-staining, 20  $\mu\text{L}$  of 1% 4,6-diamidino-2-phenylindole (DAPI) solution was spotted and incubated for 3 min. The slide was washed with 80% ethanol and distilled water to remove non-specific staining.

The specimen was mounted in immersion oil containing an anti-fading agent (Citifluor AF1, Chemical Laboratory, Canterbury, England) and observed with an Axiophot epifluorescence microscope (Carl Zeiss, Thornwood, NY). Images were captured with an Axiocam CCD (Carl Zeiss, Thornwood, NY) using the provided software. The stained cells for each probe were counted for one or two different frames. Cells that were stained with probe PB9 or probe CH1 were compared with the total number of bacteria stained with the DAPI stain.

## 2.8. Activity tests

Several weeks after collecting the last coupon, the perchlorate reduction activity was determined in R1, R2, R4, and R5. The operating conditions were similar to that of the FISH sampling. At the time of the test, R3 was no longer in operation due to a leak that developed in the fibers. For the activity tests, reactors R1, R2, and R4 were fed 5 mg N/L nitrate and 10,000  $\mu\text{g/L}$  perchlorate at 0.85 mL/min. R5 was fed 14,000  $\mu\text{g/L}$  perchlorate at 1 mL/min. The tests were carried out over 2 h, with 3 samples at 75, 100, and 125 min after initiating perchlorate feed.

## 3. Results and discussion

### 3.1. Reactor operation

At day 5, the effluent oxygen concentration in all reactors dropped to below detection (0.2 mg/L), and the nitrate in reactors R1–R4 dropped to around 30  $\mu\text{g N/L}$ . The typical effluent quality for R1–R5 was  $\text{pH} = 7.4$ , and 0.3–0.5 mg/L

dissolved hydrogen. For R1–R4, the typical effluent nitrate was 30  $\mu\text{g N/L}$ .

On day 20, perchlorate was added to reactors R2–R5. Influent and effluent concentrations are shown in Fig. 2. Perchlorate addition did not affect the effluent nitrate concentrations (not shown). One day after perchlorate addition, the amounts of perchlorate removed (influent minus effluent) in reactors R2–R5 were 27, 36, 50, and 45  $\mu\text{g/L}$ , respectively. The similar removals suggest there was a similar initial amount of PCRB in all reactors. Over the next few days, reactors R2 and R5 displayed steady increases in perchlorate removal. In reactor R2, the effluent declined to non-detect within 4 days. For R5, the effluent reached non-detect after 13 days. For R3 and R4, effluent perchlorate concentrations were around 350 and 6 mg/L, respectively, by day 27.

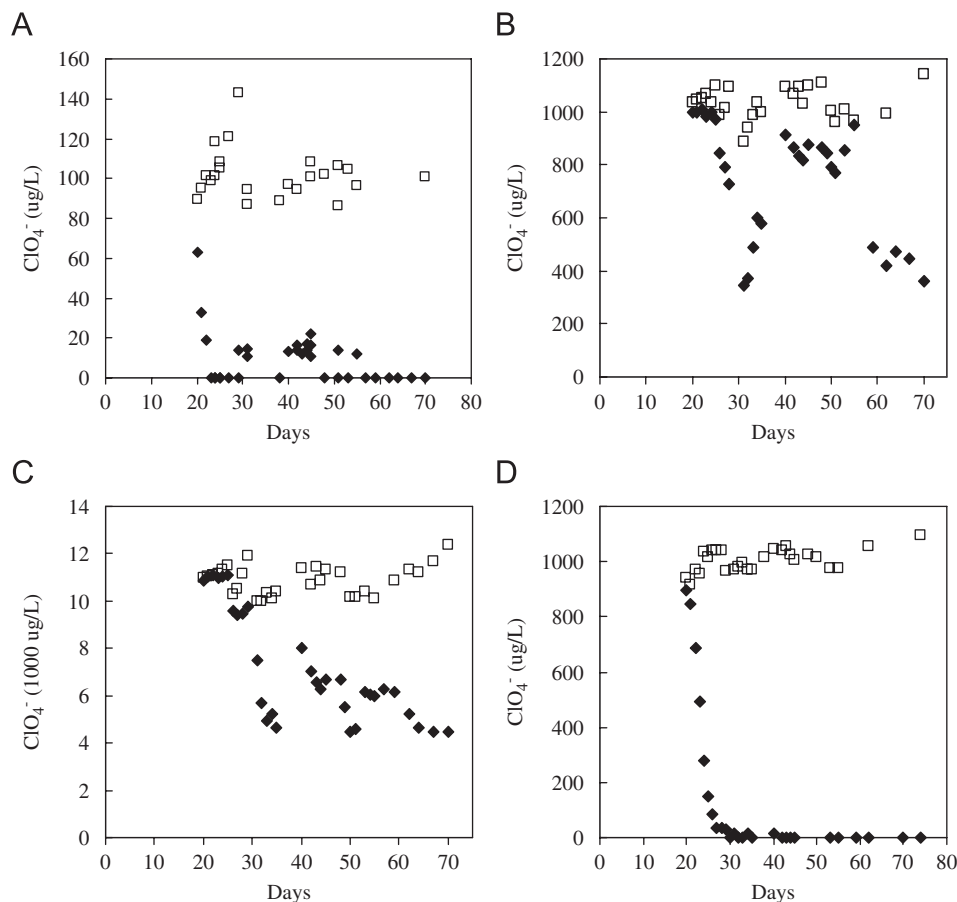
On day 28, an error in preparing the phosphate buffer for R1–R4 led to an influent pH of 6.2, instead of 7.4, and an effluent pH of 6.8. The pH excursion lasted from days 28 to 31. The pH change had no observable effect on denitrification, but it interrupted the decreasing trend of effluent perchlorate in R3 and R4. In R2, the effluent perchlorate increased to slightly above the detection limit. On day 40, the influent flow rate for R1–R4 was decreased to 0.85 mL/min to improve perchlorate removals. Within approximately 30 days, the effluent perchlorate concentrations returned to levels prior to the pH event. This result suggests that low pH is unfavorable for PCRB. This is consistent with previous research that shows that perchlorate reduction is more sensitive to pH than nitrate reduction, and that perchlorate reduction can be lost after a prolonged exposure to unfavorable conditions and recovered after favorable conditions return (Nerenberg et al., 2002).

Biofilm samples were collected for molecular analyses on day 36, 5 days after the end of the pH event, and on day 72, when effluent concentrations had returned to prior levels. Table 2 shows the influent and effluent perchlorate concentrations and removals at days 36 and 72. The percent perchlorate removals for R2 and R5 were substantially higher than for R3 and R4. The percent removals for R3 and R4 were slightly higher on day 71 than on day 36. R5 consistently reduced 1000  $\mu\text{g/L}$  perchlorate to non-detect, suggesting that oxygen may be a more favorable primary substrate than nitrate for PCRB.

### 3.2. DGGE

The DGGE results are shown in Fig. 3, with dominant bands identified with a number. In all cases, the community structures were simple and dominated by few bands. At day 20, prior to perchlorate addition, the banding patterns were similar among reactors R1–R4 (only bands for R1 and R5 shown in Fig. 3 for day 20). These had two prominent bands, 9 and 11. The strongest bands in R5 were 2 and 7, although bands 9 and 11 were also present. Despite differences in dominant bands, R5 had a similar banding pattern to R1–R4, probably because all biofilms developed with autotrophic, hydrogen-oxidizing, anoxic conditions.

At day 36, after 16 days with perchlorate addition, the banding patterns for reactors R1–R5 were noticeably different



**Fig. 2 – Influent (□) and effluent (◆) perchlorate concentrations for reactors R2 (panel A), R3 (panel B), R4 (panel C), and R5 (panel D). The influent pH for R2–R4 dropped from 7.4 to 6.2 on days 28–31. Biofilm samples from all reactors were collected on days 36 and 72.**

**Table 2 – Perchlorate concentrations and fluxes at days 36 and 71, when biofilm samples were collected**

Reactor	Influent ( $\mu\text{g/L}$ )	Day 36		Day 72	
		Effluent ( $\mu\text{g/L}$ )	Flux ( $\text{g/m}^2 \text{ day}$ )	Effluent ( $\mu\text{g/L}$ )	Flux ( $\text{g/m}^2 \text{ day}$ )
R2	100	<15	0.0126	<15	0.0143
R3	1000	580	0.0708	360	0.108
R4	10,000	4600	0.91	4500	0.927
R5	1000	<15	0.195	<15	0.195

Samples below detection ( $15 \mu\text{g/L}$ ) are taken as  $15 \mu\text{g/L}$  in calculating fluxes.

from day 20. Band 9 gained more prominence for reactors R1–R4, but band 12 was much less prominent. For R5, band 7 was prominent on day 20, but band 9 also became dominant on day 36.

All visible bands for day 36 were excised and sequenced (Table 3). The main bands for day 20, numbers 9 and 12, were most similar to *Dechloromonas JJ* (99.6% similarity) and *Rhodocyclus sp.* (95.6% similarity), respectively. For day 36, the main bands were 7 and 9. Band 7 is close to *Hydrogenophaga sp.* (96.4% similarity). Neither *Rhodocyclus* or *Hydrogenophaga* are known to include perchlorate reducers.

For day 71, after 51 days of continuous perchlorate supply, R1 and R2 had prominent bands in the 7, 9, and 12 positions, while reactors R3 and R4 had prominent bands in the 1, 2, 7, and 9 positions. For all five reactors, the most prominent band was in position number 9, most similar to *Dechloromonas JJ*. Bands 1 and 2 were only prominent for reactors R3 and R4. Although the sequence for band 9 is most similar to *Dechloromonas JJ*, a denitrifying bacterium, it is important to note that most bacteria in the *Dechloromonas* genus are PCR<sup>B</sup> and are closely related to non-PCR<sup>B</sup> (Bruce et al., 1999; Coates et al., 1999b).

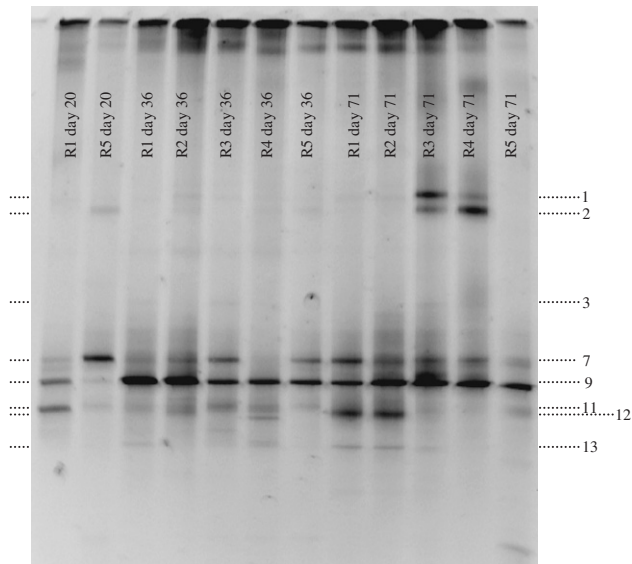


Fig. 3 – DGGE results for days 20, 36, and 71. Dominant bands are identified by number in the text.

Table 3 – Identity of dominant DGGE bands

Band	Best Genbank match	% Similarity
1	Perchlorate-reducing bacterium MLC33	92
2	<i>Chryseobacterium</i> sp.	97.1
7	<i>Hydrogenophaga</i>	96.4
9	<i>Dechloromonas</i> sp. JJ	99.6
12	<i>Rhodocylus</i> sp. HOD 5	95.6

### 3.3. FISH

Biofilm samples from day 71 were tested with two FISH probes. Probe PB9 was designed to hybridize with the band 9 sequence from the DGGE tests, and probe CH1 was designed to hybridize with strain CH1, a hydrogen-oxidizing, autotrophic bacterium previously isolated from the inoculum. Both probes, together with the DAPI stain, were applied to the reactor biofilm. Quantitative results for probe PB9 are shown in Fig. 4, which also includes results of activity tests. In R1, 14% of the total bacterial count hybridized with the PB9 probe. In R2, R3, and R4, the percentage of probe PB9 bacteria increased to 22%, 31%, and 49%, respectively. While probe PB9 hybridization correlated with perchlorate removal when nitrate was present, the actual percentage of probe PB9 hybridization was much larger than could be supported by perchlorate reduction alone. For example, perchlorate reduction could not have supported any bacteria in R1, as there was no perchlorate in the feed. Thus, nitrate reduction selected for these PCRB in R1, but the perchlorate further enriched them in R2–R5. The enrichment was beyond what would be expected from the yield on perchlorate alone. One possible explanation for the enrichment is that PCRB are more effective scavenging oxygen in the presence of perchlorate,

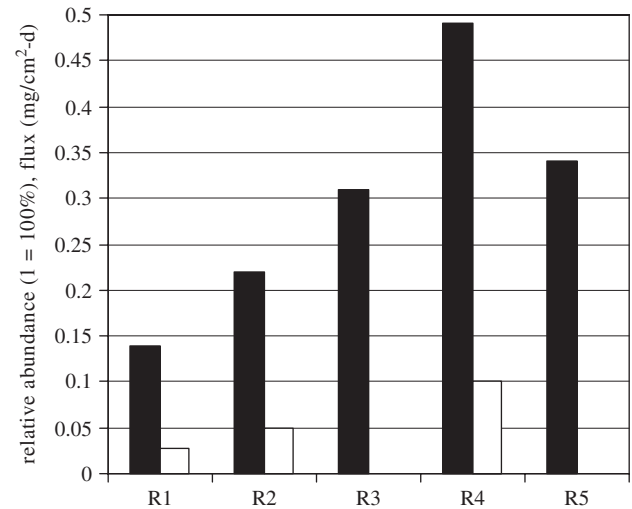


Fig. 4 – FISH and perchlorate-reducing-activity test results. The relative abundance of strain PB9 (■) is the number of PB9 counts from FISH analysis relative to total counts based on a DAPI stain, and the perchlorate reduction activity (□) is expressed as the perchlorate removal flux in mg/cm<sup>2</sup> day. The activity test was not performed for R3.

as PCRB have the unique capability of utilizing the low levels of oxygen produced from perchlorate reduction under anoxic conditions. Another possibility is that perchlorate provides PCRB with an additional source of energy, and, under the low effluent nitrate and oxygen conditions prevailing in the MBfR, they compete more effectively for nitrate and oxygen. In reactor R5, the percentage of probe PB9 hybridization was 34%, which is close to 31% for R3, which also received 1000 µg/L of perchlorate in its influent. The hybridization with probe CH1 was low for all reactors, between 2% and 6%, suggesting that perchlorate was not a strong selector for CH1. The result also is consistent with CH1 sequences not being detected in any of the reactors by DGGE analysis.

An activity test was carried out to further explore the ecology of the MBfRs on day 92. Reactors 1, 2, and 4 were challenged with a medium containing 10,000 µg/L perchlorate and 5 mg N/L nitrate, while R5 was challenged with 14,000 µg/L perchlorate. R3 was no longer operational at the time of this test. Three replicate effluent samples were collected from each reactor over three HRTs. Removal fluxes were determined based on the average concentrations from the replicates (Fig. 4). There was a slight increase in perchlorate concentration between the first and last samples, around 10% of the average value. The change is most likely due to hydraulic equilibration. These results agree with those obtained by FISH. Thus, PB9 was present in the reactor without any perchlorate, but increased in abundance, and presumably in activity, with higher perchlorate reduction.

## 4. Conclusions

This research shows that perchlorate reduction in a hydrogen-based MBfR was possible with low perchlorate

concentrations, that oxygen and nitrate served as primary electron acceptors for PCRB, and that perchlorate respiration enriched the biofilm in PCRB. Specifically,

- PCRB were present in the MBfR biofilm with oxygen or oxygen plus nitrate as primary electron acceptors, with or without perchlorate in the influent.
- When perchlorate was added to the influent, it strongly selected for the PCRB PB9, even at an influent perchlorate concentration as low as 100 µg/L. The perchlorate-reduction activity correlated with the increase in PB9.
- DGGE showed simple community structures that were similar for all reactors, even those with no perchlorate and no nitrate in the influent. These results suggest that the microbial community in the hydrogen-oxidizing, autotrophic biofilm had low diversity that was stable.
- Oxygen alone was a better primary acceptor than oxygen plus nitrate. It led to a more rapid decrease in effluent perchlorate and a lower final effluent perchlorate concentration than the MBfR with oxygen plus nitrate and the same influent perchlorate.

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