Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Quantification of genes and gene transcripts for microbial perchlorate reduction in fixed-bed bioreactors

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Keywords
biological drinking water treatment, perchlorate, perchlorate-reducing bacteria, quantitative PCR, reverse-transcription qPCR.

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2011/1347: received 10 August 2011, revised 30 November 2011 and accepted 15 December 2011

doi:10.1111/j.1365-2672.2011.05225.x

Abstract

Aims: Optimization of full-scale, biological perchlorate treatment processes for drinking water would benefit from knowledge of the location and quantity of perchlorate-reducing bacteria (PRB) and expression of perchlorate-related genes in bioreactors. The aim of this study was to quantify perchlorate removal and perchlorate-related genes (*pcrA* and *cld*) and their transcripts in bioreactors and to determine whether these genes or transcripts could serve as useful biomarkers for perchlorate treatment processes.

Methods and Results: Quantitative PCR (qPCR) assays targeting *pcrA* and *cld* were applied to two pilot-scale, fixed-bed bioreactors treating perchlorate-contaminated groundwater. *pcrA* and *cld* genes per microgram of DNA were two- to threefold higher and three- to fourfold higher, respectively, in the bioreactor showing superior perchlorate-removal performance. In a laboratory-scale bioreactor, quantities of *pcrA* and *cld* genes and transcripts were compared under two distinct performance conditions (*c.* 60 and 20% perchlorate removal) for a 5-min empty bed contact time. *cld* genes per microgram of DNA were approximately threefold higher and *cld* transcripts per microgram of RNA were approximately sixfold higher under the higher perchlorate-removal condition. No differences in *pcrA* genes or transcripts per microgram of DNA or RNA, respectively, were detected between the *c.* 60 and 20% perchlorate-removal conditions, possibly because these assays did not accurately quantify *pcrA* genes and transcripts in the mixed culture present.

Conclusions: Quantities of *cld* genes and transcripts per microgram of DNA and RNA, respectively, were found to be higher when perchlorate removal was higher. However, quantities of *pcrA* and *cld* genes or transcripts were not found to directly correlate with perchlorate-removal rates.

Significance and Impact of the Study: To our knowledge, this study represents the first application of qPCR assays to quantify perchlorate-related genes and transcripts in continuous-flow bioreactors. The results indicate that *cld* gene and transcript quantities can provide insights regarding the quantity, location and gene expression of PRB in bioreactors.

Introduction

Widespread perchlorate contamination of drinking water sources in the United States (Renner 1998) is a concern because perchlorate ingestion has adverse human health effects including thyroid disruption (Wolff 1998; Greer *et al.* 2002). The United States Environmental Protection Agency is pursuing perchlorate regulation under the Safe Drinking Water Act (USEPA 2011), and some states already regulate perchlorate in drinking water (e.g.

6 $\mu g\ l^{-1}$ in California) (CDPH 2011). Thus, reliable and cost-effective treatment options are needed. Biological treatment is a promising approach because perchlorate can be transformed into innocuous chloride and water by perchlorate-reducing bacteria (PRB), which use perchlorate as an electron acceptor under anaerobic conditions (Xu *et al.* 2003). Dissolved oxygen (DO) and nitrate are often present in perchlorate-contaminated groundwater and can inhibit perchlorate reduction and support the growth of non-PRB (Logan 1998; Chaudhuri *et al.* 2002). Thus, biomarkers are needed to assess the health of perchlorate-reducing microbial communities in bioreactors in the presence of DO and nitrate.

PRB reduce perchlorate to chlorate and chlorite using perchlorate reductase (encoded by *pcrABCD*) (Bender *et al.* 2005). Chlorite is a toxic by-product that is reduced to chloride by chlorite dismutase (encoded by *cld*). Biological perchlorate treatment has been demonstrated at the laboratory scale (Giblin *et al.* 2000; Kim and Logan 2001; Brown *et al.* 2002, 2003; Min *et al.* 2004; Choi *et al.* 2007) and pilot scale (Brown *et al.* 2008; Webster *et al.* 2009); one full-scale biological perchlorate treatment system currently operating in the United States removes perchlorate from electrodialysis reversal concentrate (Brown *et al.* 2010). To further refine full-scale processes, it is necessary to understand the impact of the type and amount of supplemented electron donor, backwashing protocols, and nutrient addition on perchlorate-reducing microbial communities. Additionally, the influence of competing electron acceptors (e.g. DO and nitrate) on the quantity and activity of PRB in bioreactors is not fully understood.

PRB quantities can be estimated using quantitative PCR (qPCR) assays targeting the *pcrA* and *cld* genes (Nozawa-Inoue *et al.* 2008; De Long *et al.* 2010), and these tools could be deployed in bioreactors to track the location and relative quantity of PRB as a function of operating parameters. However, the PRB found in bioreactors may not be actively reducing perchlorate because all known PRB are facultative aerobes, and most can use nitrate as an electron acceptor (Chaudhuri *et al.* 2002). Therefore, transcript quantities might be a better reflection of perchlorate-reducing activity, which is consistent with observations of higher quantities of *pcrA* and *cld* transcripts in cultures that were actively reducing perchlorate as compared to cultures for which perchlorate reduction was inhibited by nitrate or DO (De Long *et al.* 2010).

The objectives of this work were (1) to track the quantity and gene expression of PRB in bioreactors in the presence of competing electron acceptors as a function of bioreactor perchlorate-removal performance and (2) to determine whether *pcrA* or *cld* gene or transcript quantities correlate with perchlorate removal. To address these objectives, electron donor, perchlorate, and competing electron acceptor concentrations were monitored as a function of depth in two pilot-scale fixed-bed bioreactors that performed differently with respect to perchlorate removal and in a laboratory fixed-bed bioreactor under two distinct perchlorate-removal conditions. Previously developed qPCR assays targeting *pcrA* (Nozawa-Inoue *et al.* 2008) and *cld* (De Long *et al.* 2010) were applied to quantify genes with depth. To our knowledge, this study is the first to quantify perchlorate-related genes and transcripts in continuous-flow bioreactors.

Materials and methods

Pilot-scale fixed-bed bioreactors

Two pilot-scale, biologically active carbon (BAC) reactors (F120 and F130) were operated at a perchlorate-contaminated groundwater well in Rialto, CA (Brown *et al.* 2008; Li *et al.* 2010). The pilot-scale bioreactors had side ports to allow for liquid-phase sampling with depth. Both bioreactors had an inner diameter of 2 ft, and F120 and F130 had fixed-bed depths of 4 and 5 ft, respectively. The bioreactors were packed with F-816 Calgon granular activated carbon, and bacteria originating from the groundwater colonized the bioreactors. The influent groundwater had a pH of 7·5 and contained *c.* 50 $\mu g\ l^{-1}$ perchlorate, *c.* 6 $mg\ l^{-1}$ nitrate as N, and *c.* 8 $mg\ l^{-1}$ DO. The bioreactors were supplemented with *c.* 50 $mg\ l^{-1}$ acetic acid as the electron donor and operated at empty bed contact times (EBCTs) between 10 and 18 min. To monitor bioreactor performance, liquid samples were collected from the influent and the side ports. Perchlorate, acetate, nitrate and DO were measured as described previously (Li *et al.* 2010).

Laboratory-scale fixed-bed bioreactor

The laboratory-scale fixed-bed bioreactor consisted of four sections connected by 3-way valves to permit liquid-phase sampling with depth (Fig. 1). The column and packing dimensions were similar to those used by Choi *et al.* (2007). The inner diameter of the bioreactor was 2·54 cm; each section was packed to a height of 2·54 cm (total fixed-bed depth of 10·16 cm) with 2-mm glass beads that were etched with hydrofluoric acid as described previously (Choi *et al.* 2007). Thus, the diameter ratio of the column to the glass beads for this system was 12·7, indicating that wall effects might have affected the flow distribution.

The laboratory-scale bioreactor influent consisted of synthetic groundwater (generally based on the anion composition of the Rialto groundwater) supplemented

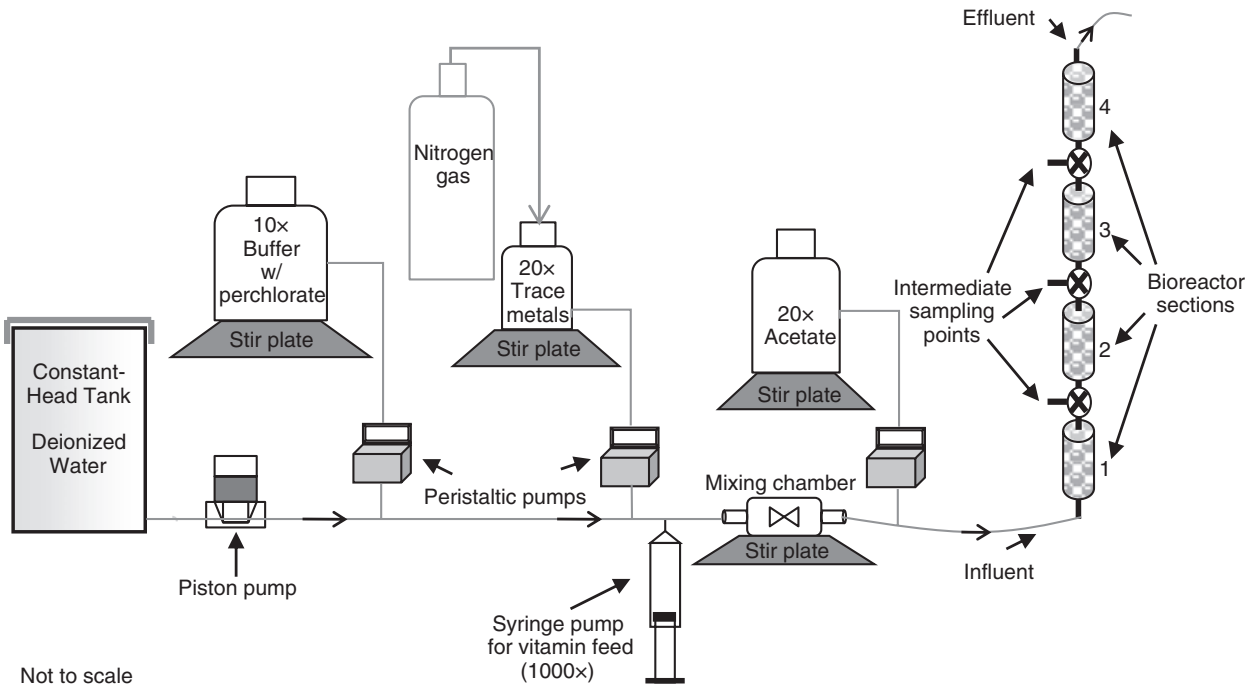

Figure 1 Schematic of the laboratory-scale bioreactor set-up.

with acetate, phosphorus, trace metals and vitamins. Perchlorate was provided at a final concentration of 100 μg l^{-1}. The influent was generated continuously by mixing concentrated stock solutions with deionized water as shown in Fig. 1. The 10× buffer contained 0·5 mmol l^{-1} K_2CO_3, 34 mmol l^{-1} $NaHCO_3$, 1·3 mmol l^{-1} Na_2SO_4, 1·0 mmol l^{-1} $CaCl_2$, 1·0 mmol l^{-1} $MgCl_2$, 4·0 mmol l^{-1} $NaNO_3$, 0·046 mmol l^{-1} phosphorus and 1000 μg l^{-1} perchlorate. The trace mineral solution was prepared as described previously (London *et al.* 2011) except that it was prepared as a 20× stock (instead of 1000×), the pH was reduced to 3·5, and the solution was sparged with nitrogen gas to prevent iron precipitation. The vitamin stock solution was prepared as described previously (Staley 1968) except that it was prepared as a 1000× stock (instead of 100×) and *p*-aminobenzoic acid was omitted. Acetate was provided as the electron donor using a 20× stock solution (2000 mg l^{-1}). After mixing, each component was present in the bioreactor influent at a concentration of 1×. With the exception of a 48-h low-DO test, the average influent DO concentration was 7·3 mg l^{-1}. The low influent DO concentration (*c.* 3 mg l^{-1}) was achieved by sparging the constant-head tank (Fig. 1) with nitrogen gas overnight.

A perchlorate-reducing enrichment culture was developed with biomass from the pilot-scale bioreactors (De Long *et al.* 2010) and used to inoculate the laboratory-scale bioreactor. The enrichment culture was pumped into the bioreactor, incubated without flow overnight and run in recirculation mode at a flow rate of 1·7 ml min^{-1} (30-min EBCT) for 22 h. After inoculation, the bioreactor was operated at a 5-min EBCT for a period of 6 weeks using the configuration shown in Fig. 1. The bioreactor was backwashed every 3–4 days by pouring the contents of each bioreactor section into a 150-ml beaker containing 50 ml of effluent and agitating gently for 1 min, similar to the study by Choi *et al.* (2007). The liquid was decanted and the beads were returned to their respective bioreactor section.

To monitor bioreactor performance, liquid samples were collected from the influent, effluent and intermediate sampling points between bioreactor sections (Fig. 1). The concentration of perchlorate was measured via EPA method 314.0 (Hautman *et al.* 1999) using a Dionex DX-600 ion chromatograph (Sunnyvale, CA, USA) equipped with AG-16 and AS-16 columns, a Dionex EGC II potassium hydroxide eluent cartridge, and a 1000-μl sample loop. Nitrate and acetate were measured using a Metrohm ion chromatograph (Houston, TX, USA) equipped with a Metrosep A Supp 5 column and a 20-μl sample loop. The eluent contained 3·2 mmol l^{-1} Na_2CO_3 and 1·0 mmol l^{-1} $NaHCO_3$. DO was measured using a YSI 54A oxygen meter with a YSI 5905 BOD probe (Yellow Springs, OH, USA) and a custom flow cell; bioreactor flow was passed through the flow cell until a steady DO concentration was measured.

Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP, as described in Kirisits *et al.* (2005), was used to characterize the microbial community in the laboratory-scale bioreactor inoculum. Briefly, DNA was isolated using the UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA), and 16S ribosomal RNA (rRNA) genes were PCR-amplified using primers 8F (FAM-labelled) and 1492R. PCR reactions were Klenow-treated to fill in partially single-stranded amplicon (Egert and Friedrich 2005). Amplicon (100 ng) was digested with one of three restriction enzymes (*Hha*I, *Msp*I, *Rsa*I) for 3 h at 37°C, and the fragments were separated on an ABI 3130 DNA analyser. The T-RFLP Analysis Program was used for putative phylogenetic identification (Marsh *et al.* 2000).

Quantifying genes and transcripts

For the pilot-scale bioreactors, vertical cores were collected with a 1-inch polyvinyl chloride pipe. Samples were frozen at −80°C prior to DNA extraction. Cells were lysed in Lysing Matrix E tubes with the FastPrep® 24 system (MP Biomedicals, Solon, OH, USA) by bead-beating twice for 30 s with the instrument set at 5·5 m s^{-1}, and DNA was isolated using the MP Bio FastDNA® Spin Kit for Soil according to the manufacturer's instructions. For the laboratory-scale bioreactor, biomass samples were collected after 26 and 37 days of operation. For DNA extraction, *c.* 50 beads with attached biofilm were removed from the top, middle and bottom of each bioreactor section (i.e. *c.* 5% of the packing was removed per section) and processed separately to extract DNA using the same procedure as for the pilot-scale bioreactors. Sterile beads were added to replace the beads that were removed. DNA isolated from the top, middle and bottom of each bioreactor section was pooled for qPCR analysis. DNA yield was quantified using the Quant-iT™ dsDNA Assay Kit, High Sensitivity (Invitrogen, Carlsbad, CA, USA).

Biomass samples for RNA extraction were collected from the laboratory-scale bioreactor as described above for DNA extraction. Cells were lysed immediately in Lysing Matrix E tubes with the FastPrep® 24 system (MP Biomedicals) by bead-beating twice for 30 s with the instrument set at 6 m s^{-1}. For the lysis step, 750 μl lysis buffer from the RiboPure™-Bacteria Kit (Ambion, Austin, TX, USA) were used, and, after lysis, RNA was purified with the RiboPure™-Bacteria Kit according to the manufacturer's instructions. Lysates from the top, middle and bottom of each bioreactor section were pooled and purified over a single RiboPure™-Bacteria Filter Cartridge. RNA yield was quantified using the Quant-iT™ RNA BR Assay Kit (Invitrogen).

To synthesize cDNA, 10 μl of total RNA was mixed with 3 μl of random hexamer primer (10 μmol l^{-1}) and 1 μl of dNTP mix (10 mmol l^{-1} of each dNTP; New England Biolabs, Beverly, MA, USA). The mixture was denatured at 65°C for 5 min and placed on ice. Four microlitres of 5× First-Strand Buffer, 1 μl of 0·1 mol l^{-1} DTT and 1 μl of SuperScript™ III reverse transcriptase (Invitrogen) were added. The reactions were incubated for 5 min at 25°C, 1 h at 50°C and 15 min at 70°C (enzyme inactivation). For each bioreactor section, duplicate cDNA synthesis reactions were conducted and pooled.

pcrA and *cld* genes and transcripts were quantified as described previously (Nozawa-Inoue *et al.* 2008; De Long *et al.* 2010). For qPCR, 5 ng of DNA were used as the template. For reverse-transcription qPCR (RT-qPCR), *c.* 5 ng of cDNA were added as the template based on measured RNA concentrations and assuming a reverse-transcription efficiency of 88% (Johnson *et al.* 2005). qPCR and RT-qPCR were conducted in triplicate from DNA or cDNA samples, respectively. The *cld* gene quantities per microgram of DNA were used to estimate the per cent of PRB in the bioreactors as described previously; *cld* was used for this purpose because the *pcrA* qPCR assay appears to undercount *pcrA* genes owing to primer mismatches with target sequences (De Long *et al.* 2010). qPCR assays also were run to quantify 16S rRNA genes as described previously (Li *et al.* 2010) as an additional means of normalizing copies of *cld* and *pcrA* genes (e.g. *cld* genes/16S rRNA gene). Genomic DNA from PRB strain *Dechloromonas aromatica* was used as the quantification standard for qPCR and RT-qPCR for all genes. The two-tailed distribution, two-sample Student's *t*-test was run to test for statistically significant differences (P-values < 0·05) in gene and transcript quantities between the two perchlorate-removal conditions.

Results

Pilot-scale bioreactor performance and gene quantification

Although DO and nitrate are generally preferred over perchlorate for use as electron acceptors (Chaudhuri *et al.* 2002; Song and Logan 2004; Nozawa-Inoue *et al.* 2005), data that delineate perchlorate removal with depth in fixed-bed bioreactors in the presence of DO and nitrate are limited (Min *et al.* 2004; Zhang *et al.* 2005). Profile data collected from the pilot-scale bioreactors F120 and F130 demonstrate that perchlorate, DO and nitrate were removed simultaneously (i.e. within the same region of the fixed-bed system) (Fig. 2). Furthermore, profile data show that the majority of the perchlorate removal

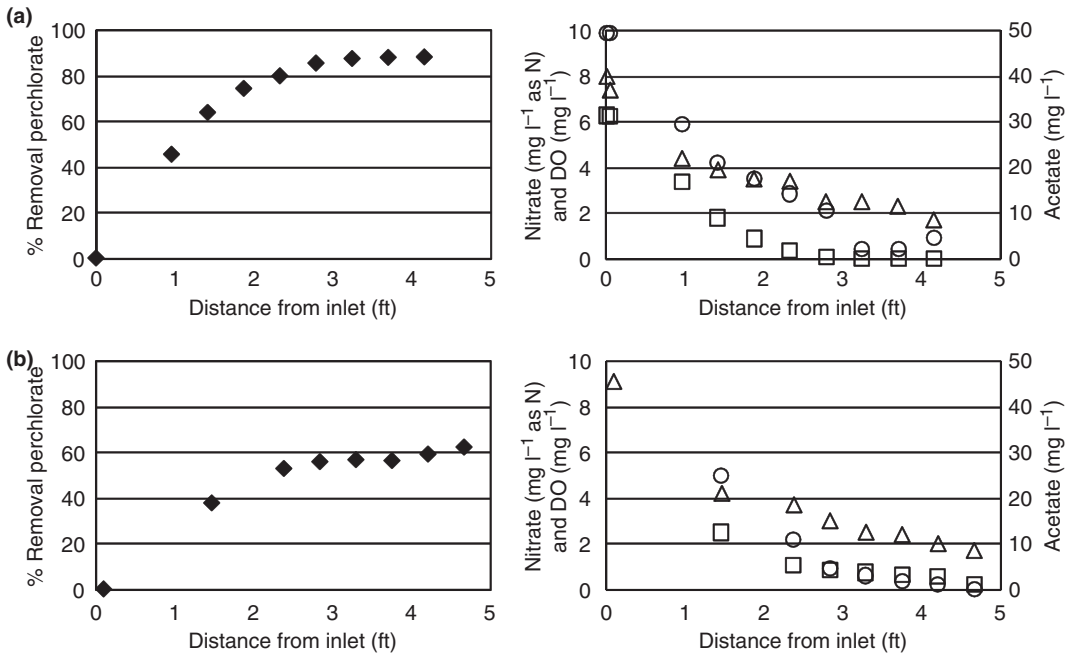

Figure 2 Bioreactor performance with depth in the pilot-scale bioreactors. (a) Profiles for perchlorate removal (◆), and DO (△), nitrate (□) and acetate (○) concentrations in bioreactor F120. (b) Profiles for F130. At the time that profile data were collected, F120 was operated at a 15-min empty bed contact time (EBCT) and F130 was operated at an 18-min EBCT. Perchlorate profiles are shown as cumulative per cent removal.

occurred in the first 3 ft of the bed, where DO and nitrate concentrations were highest. However, F120 showed greater perchlorate removal compared to F130 despite the fact that both bioreactors were supplied with the same influent, and F120 was operated at a shorter EBCT (15 min) as compared to F130 (18 min). At the time these chemical profile data were collected, perchlorate removal was 90% for F120 but only 63% for F130, and profile data show that the perchlorate, DO and nitrate concentrations were generally lower in F120 than in F130. These data show that the overall difference in perchlorate-removal performance between the two bioreactors could be attributed largely to differences in the first 5 min of EBCT (*c.* 1·3 and 1·4 ft packed-bed depth for F120 and F130, respectively). For the first 5 min of EBCT, F120 removed over 60% of the influent perchlorate while F130 removed <40% (Fig. 2).

To determine whether the differences in perchlorate-removal performance could be due to differences in the fraction of PRB in the microbial communities of the two bioreactors, *pcrA* and *cld* genes were quantified with depth in both bioreactors via qPCR. For F120 and F130, quantities of *pcrA* and *cld* genes per microgram of DNA were highest at the inlet to the bed (Fig. 3) where perchlorate removal was the highest at the time of chemical profile data collection (Fig. 2), although the bioreactor EBCTs were slightly longer at the time of chemical profile collection than at the time of biomass sampling for nucleic acid extraction (15-min EBCT as compared to a 10-min EBCT for F120 and an 18-min EBCT as compared to a 15-min EBCT for F130). Moreover, *pcrA* genes per microgram of DNA were two- to threefold higher and *cld* genes per microgram of DNA were three- to fourfold higher throughout the fixed-bed in F120 as compared to F130, indicating that a greater fraction of the microbial community consisted of PRB in F120. The per cent PRB, as estimated from the quantity of *cld* genes per microgram of DNA, ranged from 1·2 to 4·3% across the bed for F120 and 0·3 to 1·2% for F130.

Laboratory-scale bioreactor performance

To further investigate the relationship between perchlorate removal and the quantity or gene expression of PRB, a laboratory-scale fixed-bed bioreactor was operated with synthetic influent groundwater modelled after the pilot-scale groundwater. The laboratory-scale bioreactor was operated at an EBCT of 5 min, which corresponds to the region in the pilot-scale bioreactors that was most critical for overall perchlorate-removal performance. Perchlorate, nitrate, DO and acetate concentrations in the bioreactor as a function of time of operation are shown in Fig. 4. The perchlorate-reducing microbial communities present in the bioreactor were compared between two distinct

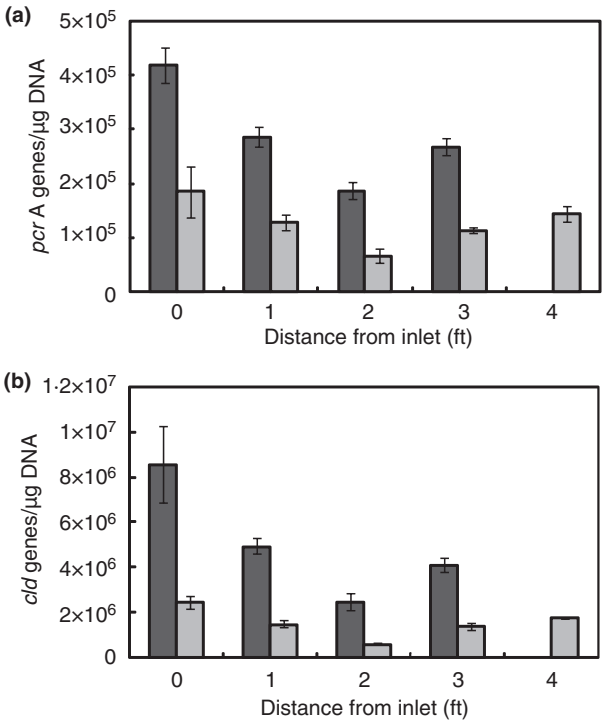

Figure 3 Quantification of *pcrA* and *cld* with depth in the pilot-scale bioreactors. (a) *pcrA* gene quantities in bioreactor F120 (dark grey bars) and in bioreactor F130 (light grey bars). (b) *cld* gene quantities in bioreactor F120 (dark grey bars) and in bioreactor F130 (light grey bars). At the time that biomass samples were collected, F120 was operated at a 10-min empty bed contact time (EBCT) and F130 was operated at a 15-min EBCT. A biomass sample was not collected for bioreactor F120 at 4 ft. Error bars represent the standard deviation of qPCR assays conducted in triplicate for a single DNA sample from each column section.

perchlorate-removal conditions, *c.* 60% perchlorate removal (day 26, labelled Profile A at the top of Fig. 4a) and *c.* 20% perchlorate removal (day 37, labelled Profile B at the top of Fig. 4a).

Although the bioreactor was operated at a short EBCT with high influent concentrations of competing electron acceptors (average concentrations of 7·3 mg l^{-1} DO and 5·3 mg l^{-1} nitrate as N), effluent perchlorate concentrations of <4 μg l^{-1} could be achieved (days 2, 14 and 21, Fig. 4a). Bioreactor profile data collected on day 21 (data not shown) and day 26 (Fig. 5a) demonstrate that perchlorate, nitrate and DO were removed simultaneously in all four bioreactor sections as was observed in the pilot-scale bioreactor. Perchlorate was actively removed even in the first bioreactor section where the influent DO concentration was as high as 7·3 mg l^{-1} and the nitrate concentration was as high as 5·4 mg l^{-1} as N. On day 26, section 1 accounted for 9% of the total perchlorate removal (where bulk DO and nitrate concentrations were higher) and section 4 accounted for 27% of the total perchlorate removal (where bulk DO and nitrate concentrations were lower) (Fig. 5a).

Although high perchlorate removal could be achieved in the bioreactor, the effluent concentrations of perchlorate, DO and nitrate increased with time between backwashing events (Fig. 4a,b), as has been observed previously (Brown *et al.* 2003). However, despite regular backwashing, perchlorate removal in the laboratory-scale bioreactor decreased over the course of bioreactor operation (Fig. 4a). Examination of perchlorate profiles on day 26 (Fig. 5a) and day 37 (Fig. 5b) showed that perchlorate removal in each bioreactor section also decreased over time. Effluent acetate concentrations under both perchlorate-removal conditions remained high, indicating that electron donor concentrations were not limiting (Fig. 4c). However, after 31 days of operation, the effluent perchlorate concentration was 70 μg l^{-1} after backwashing (Fig. 4a). At this time, the bioreactor was backwashed again, but perchlorate removal was not restored (32 days).

To determine whether the decrease in perchlorate removal was caused by a lack of PRB, the constant-head tank (Fig. 1) was sparged with nitrogen gas overnight to reduce the influent DO concentration from 7·6 to 3·0 mg l^{-1}. After reducing the influent DO concentration, the effluent perchlorate concentration was 28 μg l^{-1} (Fig. 4a), indicating that sufficient PRB were present to reduce *c.* 70% of the influent perchlorate under low-DO conditions. After nitrogen sparging was ended, the influent DO concentration was allowed to gradually increase for 1 day, and then air was bubbled into the constant-head tank to return the DO concentration to saturation. At this point, the effluent perchlorate concentration was 54 μg l^{-1} (49% removal) (day 35, Fig. 4a).

So that the quantity of PRB and expression of *pcrA* and *cld* could be examined for two distinct perchlorate-removal performance conditions (*c.* 60 and 20% removal), column backwashing was delayed for 2 days to induce a decline in perchlorate-removal performance. By day 37, no significant perchlorate removal was observed in the first three bioreactor sections (Fig. 5b), and the overall perchlorate removal declined to 23% (herein referred to as the *c.* 20% removal condition). Differences in the perchlorate-reducing microbial community between day 26 (*c.* 60% removal) and day 37 (*c.* 20% removal) were investigated via qPCR and RT-qPCR.

Quantification of genes in the laboratory-scale bioreactor

The microbial community in the laboratory-scale bioreactor likely contained both PRB and non-PRB because 16S rRNA-targeted T-RFLP analysis of the inoculum

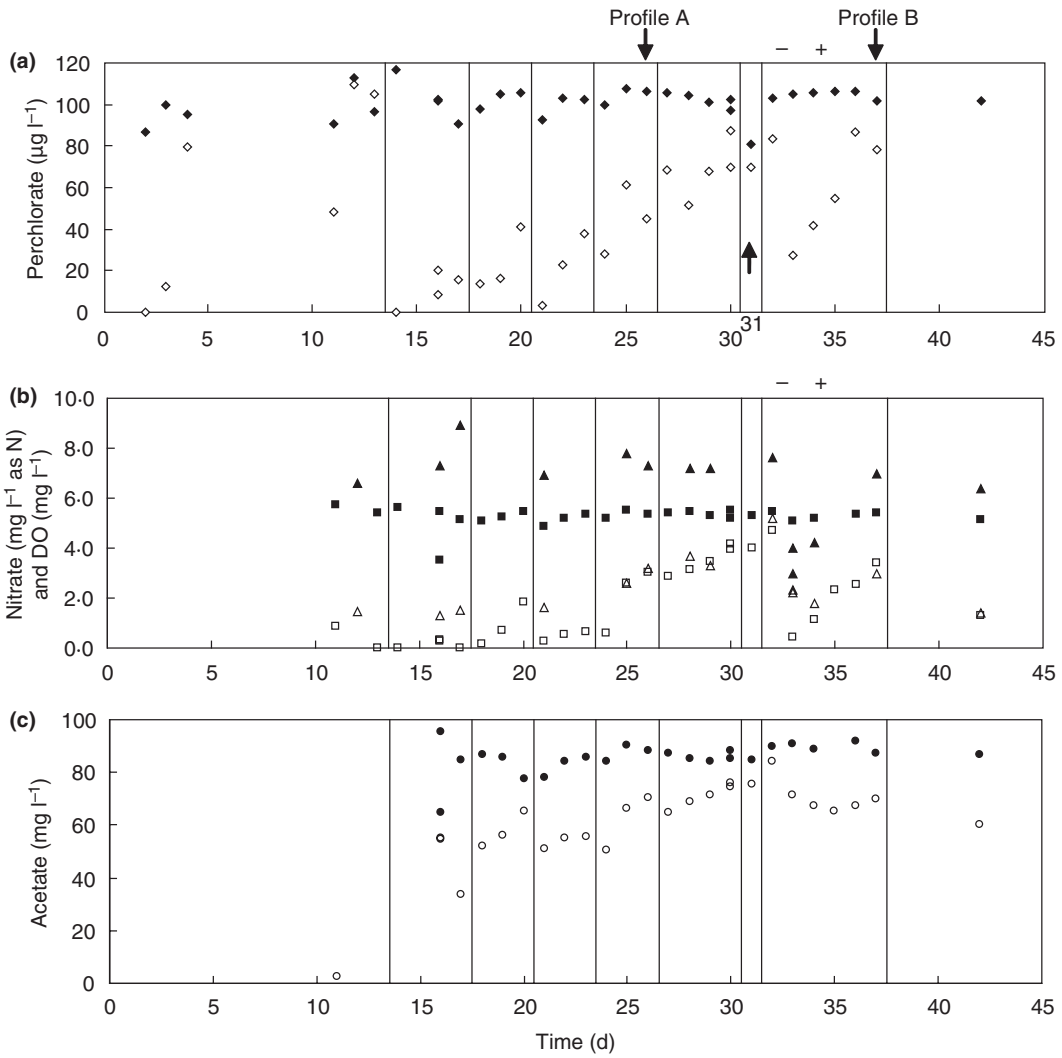

Figure 4 Laboratory-scale bioreactor performance. (a) Influent (◆) and effluent (◇) perchlorate concentrations. (b) Influent (▲) and effluent (△) DO and influent (■) and effluent (□) nitrate concentrations. (c) Influent (●) and effluent (○) acetate concentrations. Vertical lines indicate backwashes. The – and + symbols at the top of a and b indicate de-oxygenation and re-oxygenation of the influent, respectively. Arrows labelled Profile A and Profile B at the top of the figure correspond to bioreactor profiles Fig. 5a and 5b and the biomass sample collection points.

produced some terminal restriction fragments (T-RFs) that were putatively identified as *Dechloromonas* and other T-RFs that could not be attributed to any other genera of known PRB (Fig. S1). *pcrA* and *cld* genes were detected in all bioreactor sections during the *c.* 60% and the *c.* 20% perchlorate-removal conditions, indicating that PRB were present throughout the bioreactor (Fig. 6a,b). The quantity of *cld* genes per microgram of DNA did not vary much with depth in the bioreactor (Fig. 6b), suggesting that the fraction of PRB in the microbial community was relatively consistent with depth.

However, during the *c.* 60% perchlorate-removal condition, the quantity of *cld* genes per microgram of DNA and the estimated per cent PRB were two- to threefold higher than during the *c.* 20% perchlorate-removal condition (Fig. 6b and Table 1, respectively). These differences were found to be statistically significant (P-values < 0·05). Additionally, the quantity of *cld* genes per 16S rRNA gene was found to be higher in all bioreactor sections during the *c.* 60% removal condition as compared to the *c.* 20% removal condition (Fig. S2). Thus, a higher fraction of PRB in the microbial community generally correlated with higher perchlorate removal. Significant differences in the quantity of *cld* genes per millilitre of bioreactor volume between the *c.* 60 and 20% perchlorate-removal conditions were not observed (data not shown). Perchlorate-removal rate (μg min^{-1}) did not correlate with *cld* gene quantities per microgram of DNA (Fig. 7c) or with

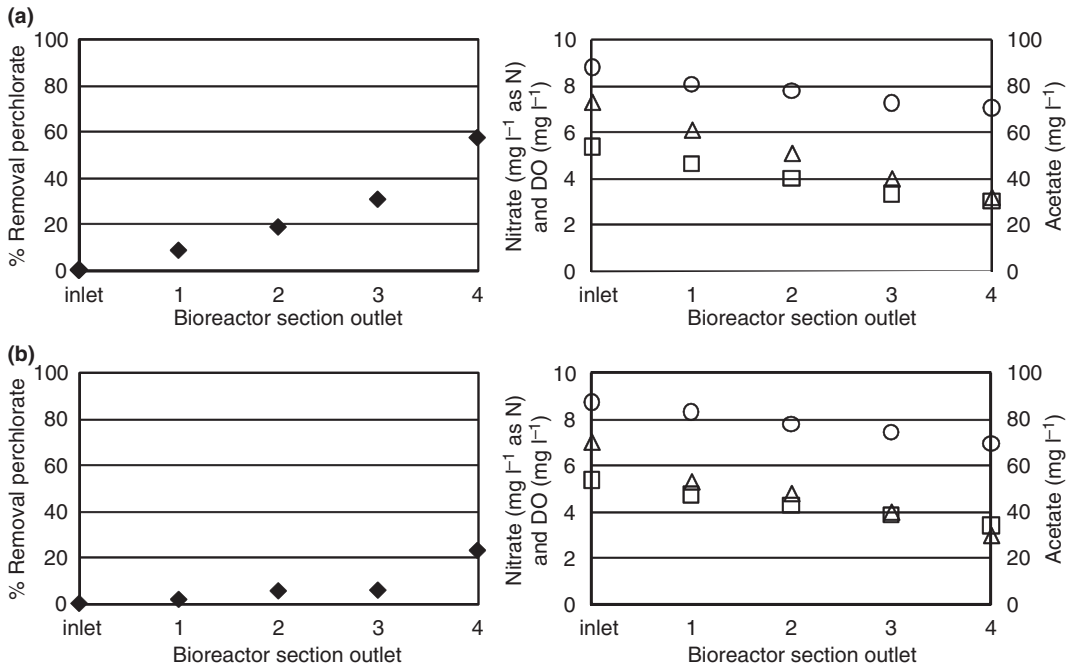

Figure 5 Bioreactor performance with depth in the laboratory-scale bioreactor. (a) Profiles for perchlorate removal (◆), and effluent DO (△), nitrate (□) and acetate (○) concentrations during the *c.* 60% perchlorate-removal condition (26 days). (b) Profiles during the *c.* 20% perchlorate-removal condition (37 days). Perchlorate profiles are shown as cumulative per cent removal.

cld gene quantities per millilitre of bioreactor volume (data not shown).

For *pcrA*, no significant change in the number of copies per microgram of DNA could be detected with depth or between the *c.* 60 and 20% perchlorate-removal conditions (Fig. 6a). Furthermore, perchlorate removal did not correlate with *pcrA* genes per microgram of DNA (Fig. 7a) or per millilitre of bioreactor volume (data not shown). However, the quantity of *pcrA* genes detected was two to three orders of magnitude less than the quantity of *cld* genes detected (Fig. 6a,b), suggesting that the *pcrA* assay did not accurately quantify PRB in this particular microbial community.

Quantification of gene transcripts in the laboratory-scale bioreactor

RT-qPCR assays were used to quantify *pcrA* and *cld* transcripts with depth in the laboratory-scale bioreactor. Both genes were expressed in all bioreactor sections during both the *c.* 60 and 20% perchlorate-removal conditions (Fig. 6c,d), even in a section where perchlorate was not actively being removed (i.e. Fig. 6c,d, *c.* 20% perchlorate-removal condition, bioreactor section 3). Interestingly, during the *c.* 20% perchlorate-removal condition, the quantity of *cld* transcripts was similar in the first three bioreactor sections and then increased slightly in section 4 where the highest perchlorate removal was observed (Fig. 6d). More substantial differences in transcript quantities were observed for *cld* transcripts per microgram of RNA between the *c.* 60 and 20% perchlorate-removal conditions (two- to eightfold higher for the *c.* 60% perchlorate-removal condition). These differences were found to be statistically significant (P-values < 0·05). Consistent differences between the *c.* 60 and 20% perchlorate-removal conditions were not observed for the quantity of *cld* transcripts per millilitre of bioreactor volume (data not shown) or for the quantity of *cld* transcripts per *cld* gene (data not shown). Perchlorate-removal rate (μg min^{-1}) did not correlate with *cld* transcript quantities per microgram of RNA (Fig. 7d) or *cld* transcript quantities per millilitre of bioreactor volume (data not shown).

The *pcrA* assay did not detect clear differences in *pcrA* transcript quantities between the *c.* 60 and 20% perchlorate-removal conditions in bioreactor sections 1–3; in section 4, higher *pcrA* transcript quantities were detected during the *c.* 20% perchlorate-removal condition than during the *c.* 60% perchlorate-removal condition (Fig. 6c). Therefore, in this system, the quantity of *pcrA* transcripts did not show a consistent correlation with perchlorate removal (Fig. 7b). As was observed for the *pcrA* qPCR assay, the *pcrA* RT-qPCR assay detected two to three orders of magnitude fewer transcripts per microgram of RNA than did the *cld* assay (Fig. 6c,d),

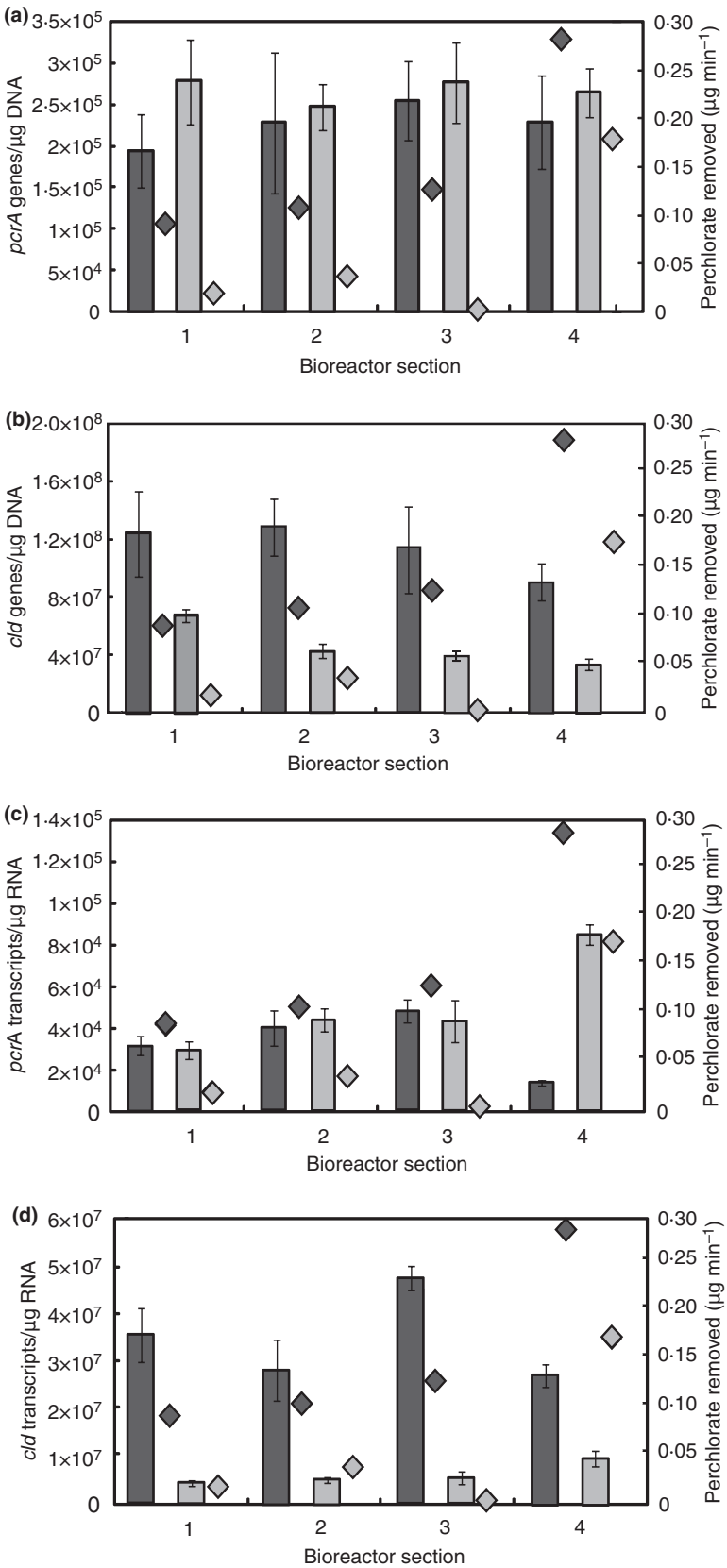

Figure 6 Quantification of *pcrA* and *cld* genes and transcripts with depth in the laboratory-scale bioreactor. Gene or transcript quantities are compared to perchlorate removal for the *c.* 60% perchlorate-removal condition (dark grey bars and diamonds, respectively) and the *c.* 20% perchlorate-removal condition (light grey bars and diamonds, respectively). (a) *pcrA* genes. (b) *cld* genes. (c) *pcrA* transcripts. (d) *cld* transcripts. Error bars represent the standard deviation of triplicate qPCR assays for the pooled DNA or cDNA samples.

Table 1 Estimated fraction of PRB (%) in the laboratory-scale bioreactor*

	Bioreactor section			
Perchlorate removal (%)	1	2	3	4
~60	31 (7)	32 (5)	29 (8)	23 (3)
~20	17 (1)	11 (1)	10 (1)	8 (1)

PRB, perchlorate-reducing bacteria.
*Estimate is based on the quantity of *cld* genes per microgram of DNA. Averages are reported for triplicate qPCR for pooled DNA samples. Standard deviations are shown in parentheses.

suggesting that the *pcrA* assay did not accurately quantify transcripts in this system.

Discussion

To guide optimization of biological perchlorate treatment processes, biomarkers would be useful to track the location, relative quantities and gene expression of PRB in bioreactors with DO and nitrate present. In the present study, qPCR and RT-qPCR assays targeting *pcrA* and *cld* were used to quantify PRB and expression of perchlorate-related genes in pilot- and laboratory-scale bioreactors containing DO and nitrate under distinct perchlorate-removal conditions.

A previous fixed-bed bioreactor study, which was operated with a high recycle ratio to simulate completely mixed conditions, showed that bulk DO concentrations above 0·2 mg l^{-1} inhibited perchlorate removal (Choi *et al.* 2007). However, the pilot-scale bioreactors in our study removed perchlorate in the presence of high bulk DO and nitrate. At a depth of 1 ft in F120, 46% of the influent perchlorate had been removed, while DO at this depth was as high as 4·4 mg l^{-1} and nitrate was 3·4 mg l^{-1} as N (Fig. 2a). Furthermore, the highest perchlorate-removal rates occurred in the bioreactor regions with the highest bulk DO and nitrate concentrations (first 1·3–1·4 ft, Fig. 2). This was likely due to the presence of a thicker biofilm at the top of the bioreactor, which we have observed in many pilot-scale systems. Perchlorate was removed in the presence of similarly high bulk DO and nitrate concentrations in the laboratory-scale bioreactor. These results suggest that thick biofilms or microbial aggregates developed in the bioreactors, and therefore, nitrate-reducing and perchlorate-reducing zones were present owing to oxygen mass transfer limitations. Laurent *et al.* (1999, 2003) observed anoxic zones inside microbial aggregates present in bulk aerobic processes. Recently, a model was developed for fixed-bed perchlorate treatment bioreactors, and this model predicted that up to 60% perchlorate removal could be achieved with a 2·7-min EBCT in the presence of bulk concentrations of 7 mg l^{-1} DO and 7 mg l^{-1} nitrate as N owing to the presence of perchlorate-reducing zones in the deep biofilm (Brown *et al.* 2008). In our study, PRB were detected throughout the fixed beds in both the pilot- and laboratory-scale bioreactors via qPCR assays targeting *pcrA* and *cld* (Figs 3 and 6). This result is consistent with the concept of perchlorate-reducing zones within the biofilm and

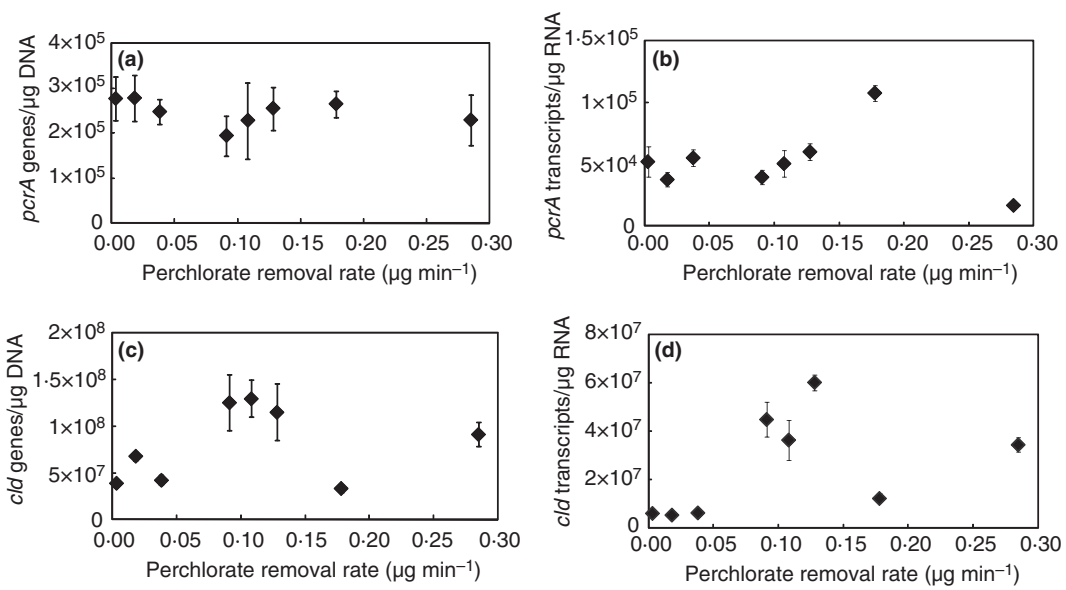

Figure 7 Comparisons between gene or transcript quantities and perchlorate-removal rates in the laboratory-scale bioreactor. The mass of perchlorate removed per minute for each bioreactor section was compared to the quantity of *pcrA* genes (a), *pcrA* transcripts (b), *cld* genes (c) and *cld* transcripts (d). Error bars represent the standard deviation of triplicate qPCR assays for the pooled DNA or cDNA samples.

the observed simultaneous removal of perchlorate, DO and nitrate.

Despite the fact that PRB were detected throughout pilot-scale bioreactors F120 and F130, and both bioreactors were treating the same influent groundwater, F120 showed higher perchlorate-removal performance than did F130 (Fig. 2). Quantification of *cld* genes per microgram of DNA indicated that the microbial community present in F120 contained a higher fraction of PRB than did F130 (Fig. 3). F130 had a deeper bed and proved more difficult to backwash as compared to F120, which might have contributed to differences in the microbial communities. The higher PRB fraction observed in the bioreactor showing superior perchlorate-removal performance (F120) suggests that the fraction of PRB might serve as a useful indicator for bioreactor health.

The fraction of PRB present in the laboratory-scale bioreactor was higher than the fraction of PRB present in the pilot-scale bioreactors (8–32 and 0·3–4·3%, respectively). This was likely due to the fact that the laboratory-scale bioreactor was initially inoculated with a perchlorate-reducing enrichment culture, while indigenous groundwater bacteria colonized the pilot-scale bioreactors. In the laboratory-scale bioreactors, PRB appeared to be using DO or nitrate as electron acceptors in addition to perchlorate because the fraction of PRB present (up to 32%) was higher than would be expected if the PRB were using perchlorate (present at low micrograms per litre levels as compared to milligrams per litre DO and nitrate levels) as their sole electron acceptor. In another laboratory-scale fixed-bed perchlorate treatment bioreactor that had been seeded with biomass from a previously operated perchlorate-reducing BAC bioreactor, Li *et al.* (2010) also found that the microbial community contained a higher percentage of the perchlorate-reducing genus *Dechloromonas* (15·2%) than could be supported by growth using perchlorate as the only electron acceptor. Similarly, Nerenberg *et al.* (2008) found more PRB than could be supported by the amount of perchlorate present in hydrogen-based membrane bioreactors that also contained DO and nitrate. Furthermore, in our laboratory-scale bioreactor, *cld* expression levels (*cld* transcripts per *cld* gene) were <1 (0·1–0·7 transcripts per gene) in all bioreactor sections, indicating that only a fraction of the PRB were expressing *cld* (data not shown). Thus, PRB likely were present in aerobic and anoxic zones or in inactive portions of the biofilm, which suggests that the quantity of PRB per unit bioreactor volume might not be a good indicator of perchlorate-removal performance.

In the laboratory-scale bioreactor, perchlorate-removal performance declined between backwashing events (Fig. 4a), likely due to a build-up of biomass that caused channelling and a decreased hydraulic residence time (Brown *et al.* 2003). It is also possible that wall effects may have led to some channelling and a decrease in performance (Choi *et al.* 2007). Perchlorate removal also declined over the course of bioreactor operation (e.g. from *c.* 60% perchlorate removal at 26 days to *c.* 20% perchlorate removal at 37 days) (Fig. 4a) possibly due to a decrease in the fraction of PRB in the biofilm (two- to threefold lower, Fig. 6b and Table 1) and a decrease in the relative expression of *cld* (two- to eightfold lower, Fig. 6d). These results suggest that over time, the laboratory-scale operating conditions (i.e. DO and nitrate present in the influent) and backwashing method (in addition to the delayed backwashing on day 35) promoted changes in the microbial community, such that the fraction of PRB decreased. Given that the bioreactor inoculum had been enriched for PRB by providing perchlorate as the sole electron acceptor, this result is not surprising. Further investigation is required to understand how perchlorate-reducing microbial communities change over time as a function of backwashing methods and bioreactor operating conditions.

The results of this study suggest that the fraction of PRB and the quantity of *cld* transcripts per microgram of RNA are likely to be the best indicators of bioreactor health with respect to perchlorate removal. In the laboratory-scale bioreactor, the estimated quantity of PRB per millilitre of bioreactor volume was not consistently higher during the *c.* 60% perchlorate-removal condition as compared to the *c.* 20% perchlorate-removal condition (data not shown). This appeared to be because the bioreactor had a higher biomass concentration under the *c.* 20% perchlorate-removal condition (as suggested by the greater mass of DNA extracted per millilitre of bioreactor volume under those conditions, data not shown). A greater quantity of biomass in the bioreactor under the *c.* 20% perchlorate-removal condition was expected because backwashing was delayed by 2 days (5 days total after previous backwash) prior to that sampling event. Although the quantity of PRB per millilitre of bioreactor volume was not correlated with performance, the fraction of PRB (as calculated from the quantity of *cld* genes per microgram of DNA) was two- to threefold higher during the *c.* 60% perchlorate-removal condition as compared to the *c.* 20% perchlorate-removal condition (Table 1). Additionally, although the quantity of *cld* transcripts per millilitre of bioreactor volume did not correlate with perchlorate-removal performance, *cld* transcript quantities per microgram of RNA were higher when perchlorate removal was higher (compare *cld* transcripts for *c.* 60 and 20% perchlorate-removal conditions in Fig. 6d).

Interestingly, under both high (*c.* 60%) and low (*c.* 20%) perchlorate-removal conditions, *pcrA* and *cld* transcripts were detected throughout the laboratory-scale

bioreactor even in sections where perchlorate removal was not observed at the time of sampling (Fig. 6c,d). Furthermore, there was no consistent difference in the quantity of *cld* transcripts per *cld* gene between the *c.* 20 and 60% perchlorate-removal conditions, indicating that the transcriptional level did not change between performance conditions. This might indicate that *pcrA* and *cld* are expressed constitutively at basal levels, which has been observed previously for *cld* in *Dechloromonas agitata* via Northern blot analysis (Bender *et al.* 2002). It is also possible that chlorite dismutase plays an unknown role in microbial metabolism in the absence of perchlorate reduction because *cld* genes recently have been identified in bacteria not known to reduce perchlorate, chlorate or chlorite (Maixner *et al.* 2008). Another explanation is that *pcrA* and *cld* transcripts might have relatively long half-lives, which has been observed previously for other environmentally relevant genes. Lee *et al.* (2006) observed that reductive dehalogenase (RDase) transcripts had long half-lives (4·8–6·1 h) and detected RDase transcripts more than 2 days after chlorinated ethenes were removed from a culture. In this case, *pcrA* and *cld* transcripts might be detected in the absence of perchlorate removal if samples were collected *c.* 2 days after the end of a period of active perchlorate reduction as occurred in the laboratory-scale bioreactor (day 35, Fig. 4a). The presence of *pcrA* and *cld* transcripts in the absence of perchlorate removal might indicate that the perchlorate-reducing microbial community would recover more quickly from stressful conditions (e.g. DO spikes) because translation of perchlorate reductase and chlorite dismutase transcripts could resume immediately after stresses were removed without *de novo* transcription. For PRB in mixed microbial bioreactors, further research is needed to determine whether *pcrA* and *cld* are constitutively expressed at basal levels or whether their transcripts have long half-lives.

Although *cld* transcript quantities per microgram of RNA were higher when perchlorate removal was higher, the rate of perchlorate removal was not correlated with the quantity of *cld* transcripts (Fig. 7d). Given the potential inaccuracies with the *pcrA* qPCR assay in this system, it is unclear whether *pcrA* transcripts are correlated with perchlorate removal. To evaluate such correlations, the database of *pcrA* gene sequences needs to be expanded to allow for the development of an improved *pcrA* assay. Because currently available *pcrA* sequences exhibit substantial divergence, the availability of additional sequences is necessary to guide the design of improved *pcrA* qPCR consensus primers (De Long *et al.* 2010). However, if perchlorate reductase and chlorite dismutase activities are regulated translationally or post-translationally, there might not be a correlation between perchlorate removal and quantities of *cld* or *pcrA* transcripts. Previous studies have found that transcript quantities do not necessarily correlate with degradation rates. Lee *et al.* (2006) did not find a correlation between RDase transcript quantities and dechlorination rates, although transcript quantities were generally higher during dechlorination of chlorinated ethene. If a correlation between *pcrA* or *cld* transcript quantities and perchlorate-removal rates does not exist, further research might aim to identify a threshold level of *cld* transcripts per microgram of RNA that is indicative of a healthy perchlorate-reducing microbial community in continuous-flow bioreactors.

In this study, quantification of *pcrA* and *cld* genes and transcripts provided insight regarding the location, relative quantities and relative gene expression levels of PRB in bioreactors for drinking water treatment. The results of this study indicate that *cld* could be a useful biomarker for perchlorate removal. Relative quantities of *cld* genes and transcripts could be compared for different treatment processes and operating conditions (e.g. backwashing methods) to identify conditions that lead to higher fractions of PRB or higher levels of *cld* expression. qPCR and RT-qPCR assays can readily be applied to a large number of bioreactor samples in a relatively short time (*c.* 8–10 h), making them ideal for analysing mixed microbial communities over a wide range of operating conditions. Thus, these assays could be applied to gain insights and guide the optimization of biological perchlorate treatment processes.

Acknowledgements

We thank David Wahman, David Stump and Ramakrishnan Kannappan for help with the laboratory-scale bioreactor design and set-up as well as Gerald Speitel, Jr., George Georgiou and Shelley Payne for their insightful comments. We thank Greg Estavo and Axel Ettori of Carollo Engineers for obtaining samples from the pilot-scale bioreactors. The pilot-scale demonstration project was supported by the U.S. Department of Defense Environmental Security Technology Certification Program (W912HQ-06-C-0020 to J.B.). The bench-scale research was supported by the U.S. Department of Defense, through the Strategic Environmental Research and Development Program (SERDP, W912HQ-07-P-0039 and W912HQ-10-C-0056 to M.J.K.), and a scholarship from the Environmental Research and Education Foundation (to S.K.D.).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Microbial community profiling by T-RFLP for the bioreactor inoculum.

Figure S2 Quantification of *cld* genes per 16S rRNA gene with depth in the laboratory-scale bioreactor.

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